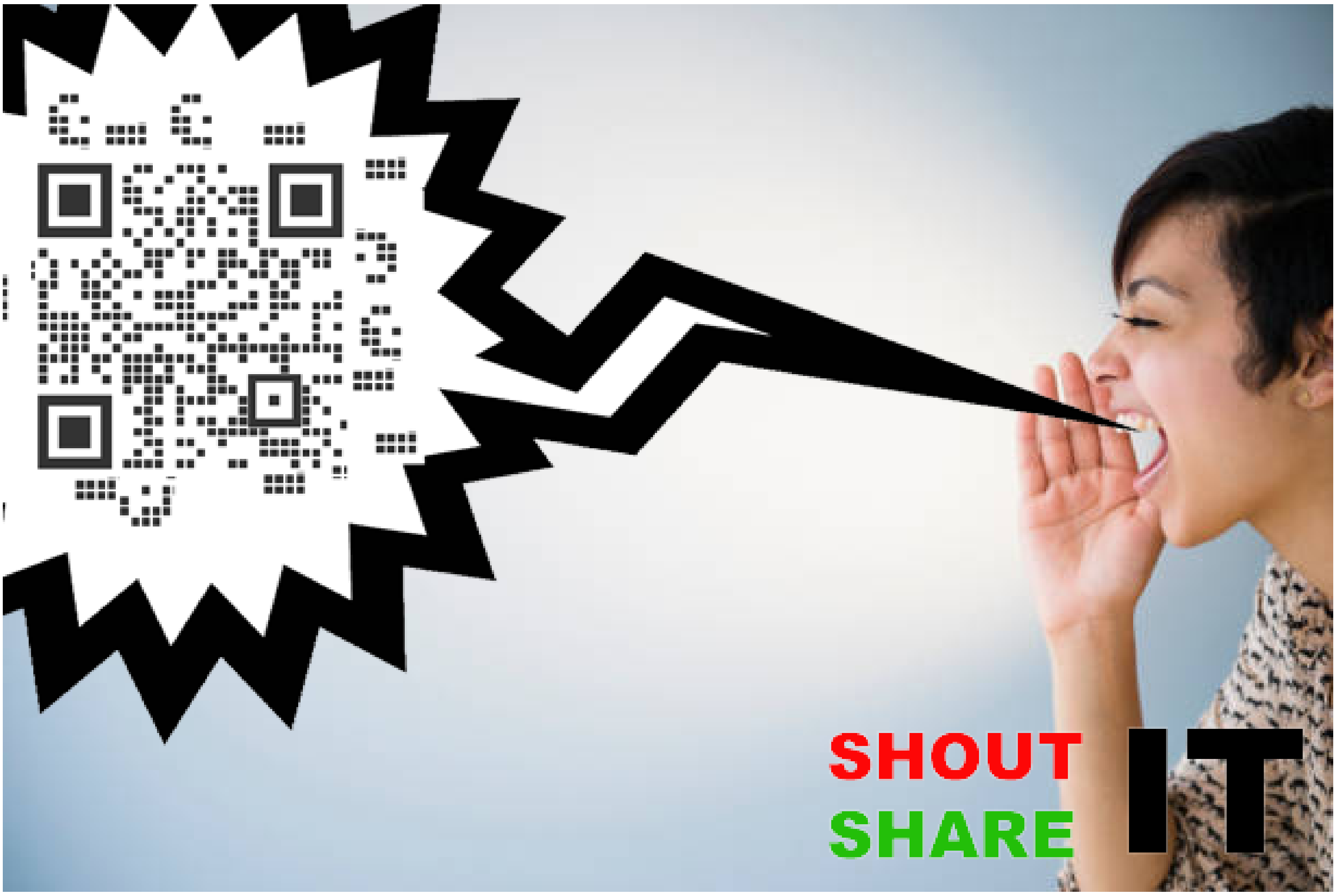
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BIOLOGICAL BULLETIN

OF THE

Marine Biological Laboratory

WOODS HOLE, MASS.

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BIOLOGICAL BULLETIN

ON THE GEOTROPISM OF PARAMECIUM AND SPIROSTOMUM.[1]

SAKYO KANDA,

DEPARTMENT OF PHYSIOLOGY, UNIVERSITY OF MINNESOTA.

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I. INTRODUCTION.

Through the kindness of Professor Ralph S. Lillie, I was given an opportunity to study in the physiological department of the Marine Biological Laboratory at Woods Hole, Mass., during the summer—July 1–September 4—of 1912. During my stay the following experiments were made under the direction of the instructors in the physiology course, especially of Professor E. P. Lyon. I wish here to acknowledge my indebtedness to Professors Garrey, Knowlton, Lillie, Lyon and Meigs.

The general phenomena of geotropism in the protozoa are well known through the work of Schwarz (22), Aderhold (1), Massart (18), Jensen (11–12), Sosnowski (23), Moore (19), Jennings (5–10), Lyon (16–17), Harper (3–4), and others. The description of the phenomena given in these papers is exhaustive and no recapitulation is needed.

In this paper an attempt has been made to subject to a critical and experimental reëxamination the various theories of geotropism in the protozoa, especially in *Paramecium* and *Spirostomum*. The experimental work has been largely confined, with variations, to the repetition of the work of Lyon and Harper.

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[1] From the Marine Biological Laboratory, Woods Hole, Mass.

As is well known, paramecia are extremely sensitive to chemicals or food, to contact stimuli, to water currents, to mechanical agitation, to changes of temperature, etc. For geotropic experiments, therefore, the sources of those stimuli must be carefully excluded. If this has been done and if paramecia washed in pure distilled water are placed in a vertical *clean* glass tube, they swim upward and gather at the top. In other words, paramecia under proper conditions are negatively geotropic. But Sosnowski (21, p. 131), Platt (20, p. 32), Jennings (9, p. 473; 10, p. 76), Lyon (17, p. 421), Harper (3, p. 995) and others have found considerable variation in the behavior of individuals in different cultures; some orient themselves downward and swim in that direction, while others are apparently indifferent to gravity. Nevertheless, in the majority of cases there is no doubt that gravity is the real "directive force." We are thus confronted by the established fact of negative geotropism in paramecia, but different investigators offer varying explanations for the phenomenon.

II. THE MECHANICAL THEORY.

From his study of the reactions of *Euglena* and *Chlamydomonas* to gravity, Schwarz in 1884 reached the conclusion that the negative geotropism of these animals is due to the direct influence of gravity on the organisms, inciting movement in the opposite direction (22). Aderhold in 1888 investigated *Euglena* (1, pp. 317–320) and *Desmid* (1, pp. 339–340), and came to the same conclusion as Schwarz.

Though he seems now to accept the "statolith theory" (25, pp. 526–527), Verworn claimed at first that the effect of the force of gravity exerted upon the protozoa is "purely physical" (24, p. .121). According to him, "the protoplasmic mass of greater specific gravity sinks toward the bottom . . . , while that of less specific gravity rises toward the surface and stays in that position." On such a "purely physical ground," he criticized Aderhold and believed it self-evident that "in complete quiescence of the flagellum the posterior end of the protist should be directed downward and not the flagellated anterior end." "With its flagellum directed upward and moving it, such an oriented individual must move toward the surface of the water" (24, p. 122). Further, Verworn believed that the force of gravity does not act upon the organism as a stimulus. This view has been called by Davenport the "mechanical theory" (2, p. 121).

After experiments on bacteria, flagellata, and ciliata, Massart in 1891 arrived at the same conclusion as Schwarz and Aderhold; and he stated that his "experiments do not agree with Verworn's theory" (18, p. 166).

After a more extensive and careful study than any of his predecessors had made, Jensen in 1893 "disproved" the mechanical theory of Verworn and proposed his pressure theory instead. But Jensen's experiments were not beyond criticism for his methods were unsatisfactory. He "killed" 50 paramecia in a "filtered alcoholic iodine solution" and watched the individuals fall in the solution to determine which end, anterior or posterior, was directed downward. More than half of them, according to him, sank without any definite orientation of the axis or in "beliebiger Querstellung," while a greater part of the rest sank with their posterior ends directed downward, and the other part with their anterior end directed downward.

From these results and others, he concluded that "the orientation of the *Paramecium* with the long axis vertical and in the direction of gravity would not be brought about by a purely physical effect" (11, p. 455). Jensen also tried similar experiments with *Euglena viridis*, and found that the majority of them sink with their anterior end downward (11, p. 457).

Recently Lyon rightly criticized Jensen's experiments in killing the animals in the alcoholic iodine solution, saying that "the centers of buoyancy and gravity might have been changed through distortion or through localized changes of density in the protoplasm" (17, p. 423). Lyon having questioned Jensen's method, "made use of the fact that the animals when centrifuged strongly are passively thrown to the bottom of the tube." "A glass tube was drawn into a capillary so fine that paramecia could not turn around in it. The capillary end was dipped into distilled water and the tube filled by capillarity . . . ; then the end was sealed and a drop of water containing paramecia was introduced into the large part of the tube. The tube was fastened to the centrifuge so that the capillary pointed away from the axis of the machine. After strong rotation the capillary was examined under the microscope. The organisms were invariably found with anterior ends directed towards the closed end of the tube." From these results, Lyon concludes: "It is therefore certain that passive paramecia would fall head end down, and that their negative geotropism is the result of an active process on the part of the animals. Jensen's view in this respect is fully confirmed and the mechanical theory is to be laid entirely aside" (17, p. 423).

Judging from Lyon's results, the anterior end seems to be "heavier" in spite of the larger shape of the posterior end. Thus Verworn's theory as well as Davenport's supposition of a static equilibrium (2, p. 122) are found untenable.

Very recently, however, Harper has taken up the problem and in his two articles has again revived the "mechanical theory" (3–4). He adopted the method of Kreidl (13) and that of Prentiss (21) who made crustaceans take in iron-filings as "statoliths" and then applied a magnet. Harper made *Paramecium caudatum* ingest finely divided iron prepared by the alcohol method. In so doing he varied the time for the ingestion of iron, from 30–40 seconds to five minutes. He seems to have obtained his best results when the ingestion of iron was allowed to continue for one minute. "The treated animals," he says in his first article, "swarmed to the top *more quickly* and formed a denser ring there than in the control" (p. 996). From this result and others, Harper drew the conclusion that "the pull of gravity on the *heavier* posterior end may produce a tipping effect which is able to *orient passively but is too weak to stimulate*."[1] And he believes that here "we have in the normal, quiet, geotropic reactions of *Paramecium* an example of a *purely mechanical* tropism"[1] (3, p. 998).

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[1] All italics not in the original.

Harper also claims that the posterior end of normal paramecia is heavier, because they assume the same position in respect to gravity as do those paramecia which have ingested iron in their posterior ends, and which are therefore undoubtedly heavier. But a generalization from such an experiment is not always safe. Spiders, for instance, whose posterior region or abdomen is unmistakably larger and heavier than the anterior region, always orient the head downward *at rest*.[1] Through his experiments, therefore, Harper is hardly justified in drawing his conclusion. Again, according to him, there is no negative geotropism of an active kind on the part of the normal paramecia, though Lyon thinks there is, because the "pull of gravity" "is too weak to stimulate," unless it is augmented by "a greater force" like a strong centrifugal force. "When, however, a stronger force is substituted for gravity so as to produce a sudden orientation, the irritability is affected, and the animal reacts to the change" (3, p. 999).

This conclusion was based on the rheotropism of paramecia. "When . . . the centrifugal effect," he says, "exceeds the pull of gravity and produces too sudden an orientating tendency, this may act as in the ordinary rheotropic reaction against a current. When strongly centrifuged the animal takes a position so that it moves in the water just as the water moves past it in the rheotropic response." "If it allowed itself to be oriented by the centrifugal force with posterior end in advance its relation to the water would be the reverse of what it is in the rheotropic response" (3, p. 998).

From this point of view Harper criticizes Lyon's results. According to the former, "the inference that the anterior end is heavier is contrary to what the shape of the body would indicate, unless the heavier particles are located anteriorly." So he suggests "as an explanation of Lyon's experiment that in strong centrifugalization the same effect is produced at the outset as by mechanical agitation, *i. e.*, the reaction changes to positive." After a repetition of Lyon's centrifugalization experiments,

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[1] Moreover, the larvæ of a marine annelid, *Arenicola*, the anterior end of which is larger and heavier than the posterior end, are negatively geotropic. The writer has found by centrifuging that its anterior end is heavier than the posterior end, and further experiments are still in progress (1913).

"the explanation here given" "seemed to him the most satisfactory explanation of the fact that all are found to move with the anterior end outward" (3, p. 998). This suggestion of Harper is worth serious consideration as a criticism of Lyon's experiments, and must not be overlooked; because we know from Sosnowski (23, p. 133) and Moore (19, p. 243) that paramecia which have gathered at the top of a tube often become positively geotropic by a "shock" or "mechanical agitation." The present writer also found this to be true. But two series of questions arise: (*a*) What makes the animals swim downward after the mechanical shock or agitation? If the "pull of gravity" "is too weak to stimulate," it should not produce a positive geotropism any more than a negative one, because the pull of gravity always remains the same before and after the mechanical shock. In Lyon's centrifugalization experiments, however, the "substituted" centrifugal force continued to act until the animals were carried into the capillary tube where they could not turn around. Centrifugalization and temporary mechanical agitation have thus different effects. And (*b*), if the posterior end of the animals is "heavier" than the anterior end, as Harper claims, is it possible that the animals, when *strongly* centrifuged, are really "able to react at the outset of centrifugalization," so as to direct themselves with the "lighter" anterior end away from the axis of the centrifuge? If we believe in a mechanical explanation in this case, as Harper does in the normal case of the "passive orientation," we should hardly expect that this would be the case, because Lyon employed too strong a centrifugal force and the mechanical effect is proportioned to the force acting. It seems to us therefore that the question remains unsettled, though Harper seems satisfied with the mechanical theory.

In his second series of experiments, Harper used a strong electromagnet applied to the iron-ingested paramecia. From the results, he concludes that "the passive sinking of the posterior end is able to orient the animal into a position of gravity equilibrium with the anterior end up" (4, p. 189). As to the magnetic effect, he thinks that it caused in the iron-ingested animals "an upward streaming in the stronger part of the field. Those heavily loaded animals which move upward in this stream after dispersing above into the weaker part of the magnetic field tend to sink again and cause a return circulation to the bottom. The magnet is effective in producing this circulation by diminishing the effect of gravity on animals containing iron. It also exerts a passive pull upon them and they gradually swing into their finally oriented position in a vertical path under the combined influence of the magnet and gravity" (4, p. 189). Harper could not get any definite results showing a direct effect of the magnet. "The movement toward the magnet," he says, "is the most diffused feature of the circulation, so that superficially it might be set down as the result of the random movements of the animals" (4, p. 185).

1. *Experiments on Paramecium caudatum.*

Experiment a.—Judging from the results of both his experiments, Harper contributes little to the solution of our problem, except his criticism on Lyon's experiments already mentioned. Hence the present writer looked for a method that would obviate Harper's objection to Lyon's conclusions as much as possible. He took advantage of the fact that the paramecia are positively rheotropic, that is, they show the positive reaction to water currents as already stated. For this purpose, he repeated Lyon's method of centrifugalization with a capillary tube like that described by Lyon. A glass tube about 4 mm. in diameter and 7 cm. in length was drawn at the middle into a capillary tube so fine that paramecia (and spirostoma) could not turn around in it.

The parts of *abc* and *cde* were both about 3.5 cm. long. The end of *e* was "sealed" in Lyon's case, but it was *left open* in those used by the writer for the following reasons. The heavier ends of the animals, when centrifuged in the tubes so prepared, are not only passively thrown to the end of the tube away from the axis of the centrifuge so that they close the open end of the tube themselves, but at the same time the current of water is also thrown in the same direction with the animals. The animals being positively rheotropic, as Jennings found (9, pp. 468–473),[1] they tend naturally to orient themselves in the tube with their anterior end toward the axis of the centrifuge as far as their rheotropism is concerned; thus against the current. Therefore, to some extent, this positive rheotropism, when the animal is centrifuged, will tend to prevent the downward, *i. e.*, positive "orientation" of *Paramecium* to gravity "at the outset of the centrifugalization" as by mechanical agitation. In such a case, if the posterior ends of the animals are "heavier" than their anterior ends, as is supposed by Harper, it would be expected that their posterior ends would be passively thrown to the end of the tube *away from* the axis of the centrifuge while the anterior ends would be "oriented" against the water current *toward* the axis. Thus this method obviates in a measure Harper's criticism rendered on Lyon's experiment. Now the writer put some distilled water in the part *bc* of the tube (see diagram), say about 2.5 cm. long, and then added by a small pointed pipette two or three drops of a dense suspension of paramecia, absolutely free from sediment, in the part *ab*. The tube was fastened to the centrifuge as soon as possible, so that the capillary end when centrifuged should be pointed away from the axis of the machine. After centrifuging at a rate of about fifteen revolutions per second for 10 seconds, the capillary was examined under the low power of the microscope. Every animal was without exception found with its anterior end directed toward the open, outer end of the capillary tube in every trial. This was carefully repeated, varying the speed of the centrifuge, that is, with both higher and lower speeds than 15 revolutions per second. The amount of distilled water in the part *bc* was also varied from a column 2.5 cm. long to zero. But this made no difference.

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[1] The present writer also found that their positive rheotropism is often stronger than their negative geotropism.

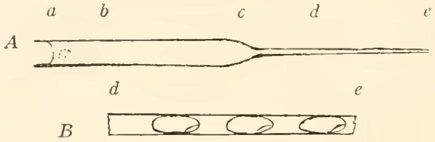

FIG. 1. Tube *A* ready for centrifuge, and *B* a part of capillary after centrifuging, magnified, showing paramecia forced down head first (a slightly modified Lyon's preparation).

The writer calculates the centrifugal force which he used for the above experiments as follows: The revolutions with the radius about 4 cm. long being about 15 per second, the centrifugal force is given by the formula

$$f = M \cdot \frac{(2\pi r \cdot n)^2}{r},$$

where M = mass in grams, r = radius of circle, n = number of revolutions per second. This gives for $r = 4$ cm. and $n = 15$, $f = M \cdot 35{,}494$ dynes or $M \cdot 36.2$ grams. Moreover, the revolutions were increased to about 20 per second, more or less, as already stated, and then the centrifugal force was about $M \cdot 59{,}101$ dynes or $M \cdot 60.32$ grams. Under these conditions, is it thinkable that such a small animal as *Paramecium* "is able to react at the outset of centrifugalization" against the centrifugal force of the above calculation so as to direct itself with its "heavier" posterior end toward the axis of the centrifuge as Harper claims?

Moreover, Harper states that "an excessive amount of iron may *overload*[1] the animals apparently and caused them to aggregate at the bottom" (3, p. 995). Even though an "overload of iron" might be *quite excessive* for the animal, it could not possibly be comparable with the centrifugal force which the writer used. Nevertheless, Harper thinks that in the case of the *strong* centrifugalization "the animal is able to react" with the supposedly lighter anterior end directed away from the axis of the centrifuge. Such mechanics we can hardly understand.

Experiment b.—With the same point in mind, the writer made another series of experiments with iron-ingested paramecia, as suggested by Harper's experiments. He mixed about a half-gram of finely divided iron prepared by the hydrogen method with about 2 c.c. of a dense culture of paramecia free from sediment. The iron was kept in suspension by drawing the water in and out with a pipette for about 5 minutes. All the paramecia so treated ingested iron in the posterior end of the body. Two or three drops of the culture containing paramecia so prepared were put in a tube as described in the preceding experiments; the tube was already filled between the points *bc* with about 0.2 c.c. of distilled water. The tube then was centrifuged. The procedure was exactly the same as the preceding experiment. In this case, every animal was found with its posterior end unmistakably directed toward the open outer end of the capillary tube. From the presence of the ingested iron in the posterior end of the animals, we should expect this end to be *heavier* than the anterior end, as Harper says. It is no wonder then that the "heavier posterior end" in this case was passively thrown away from the axis of the centrifuge, as the heavier anterior end was in the normal case. This is the only possible conclusion we can draw. Lyon's view in this respect is fully confirmed, and Harper's explanation is not satisfactory.

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[1] Italic not in the original.

Experiment c.—The writer repeated Harper's experiments on the paramecia which had ingested iron as already described. He placed one control tube containing a thick culture of normal paramecia, side by side with a series of five tubes, *a* to *e*, containing iron-ingested animals which were exposed to the iron-containing medium for different periods, as follows: *a* for 30–40 seconds, *b* for two minutes, *c* for 3.30 minutes, *d* for 5 minutes, and *e* for 10 minutes. All of the tubes were in a vertical position. About 18 minutes later, in the control tube, about 70 per cent. of the animals had gathered at the top, 20 per cent. at the bottom, and the rest were scattered through the tube. In all the treated tubes, about 80 per cent. of the animals had gathered at the top and the rest were scattered through the tubes; except in tubes *a* and *b* where there was on the bottom of each tube a small dense gathering which disappeared about 10 minutes later. After about two hours and fifty minutes, in all treated tubes, almost all the animals had gathered at the top, especially in tube *e*, and a very few were scattered here and there, while in the control, 70 per cent. were still at the top and the rest at the bottom.

According to Harper, the ingested iron "particles are at first lodged in the posterior end," and "the changed response is coincident with this condition and tends to disappear as the particles become more evenly distributed through the endoplasm" (4, p. 181). The writer continued his observations for about three hours and found that no such change in reaction occurred. At the end of the time, almost all the animals in his case were at the top of the tubes.

About 16 hours later, in the treated tubes, all the paramecia were dead except a few in tubes *a* and *b*. They may have died earlier than this, as the writer did not have a chance to observe them in the meantime. He examined the dead animals under a microscope, but the iron particles were not noticeably "distributed through the endoplasm"; some animals were found vacuolated and many must have broken into pieces, because few dead animals were found. On comparing the above observations with Harper's, we find some differences. According to Harper, "the paramecia rid themselves of the iron after the course of a few hours without apparently harmful effects from a small amount" (3, p. 995). It may be premature to assume a chemical effect on the protoplasm of the iron-ingested paramecia, but we may expect some abnormal reactions of the animals, besides the purely physical; because we saw that all the paramecia treated for 3.5, 5, and 10 minutes died within at least 16 hours, as already stated. In other words, the more iron the animals ingested the quicker their death resulted.

From the above results it is clear that the iron-ingested paramecia are more apt to be negatively geotropic than the normal ones, as Harper has shown. Since the iron is ingested in the posterior region, which presumably is thus made heavier than the anterior region, Harper's view of a "passive orientation" may be true in this particular case. The writer does not mean, however, by "passive orientation," as Harper does, that the "pull of gravity" "is too weak to stimulate" in any case. In point of fact, the normal *Paramecium* orients its anterior end upward not because that end is heavier but *in spite* of it. Of course, therefore, when the posterior end is rendered heavy by iron the orientation is easier. It is here that the "mechanical theory" deserves some credit. It gives us one of the factors of orientation of some animals—like iron-ingested paramecia—against the force of gravity. The "stern-heaviness" or "bow-heaviness," therefore, may be one of the conditions of stimulation which bring about orientation. But it is not the only condition because, as we have already observed, the negative geotropism of the normal paramecia may be altered by a mechanical agitation to a positive geotropism. We must, therefore, hold with Moore (19, pp. 243–244), Jennings (10, p. 99), and Lyon (17) that there are some additional internal or physiological conditions of orientation in such a case.

Experiment d.—In the experiments on iron-ingested paramecia the writer used an electromagnet not as strong as that used by Harper, employing a binocular microscope placed horizontally for the observations of the movements of the animals, so adjusted that the experimental tube was readily observed. The writer found in these experiments that many animals swam upward, while others swam downward, and a few others toward and away from the electromagnet, which was applied at one side of the tube. According to Harper, the path of paramecia so treated represents the resultant of the pulls of gravity and of the electromagnet. The writer saw some behave in this way, but he could hardly specify it as characteristic. He was unable to detect any orientation with respect to the magnetic force. Possibly the weak electromagnetic force in the writer's case might be the cause of the difference between his results and those of Harper. But even with such a weak force it was observed in many cases that the pull of gravity was easily obscured by the pull of the electromagnet. In a few cases it was observed that the individual when passing in front of the magnet was passively drawn backward toward it when the electric current was connected and moved in a straight line away from it when the current was disconnected. Such cases indicate that the pull of gravity has no effect on the animal in comparison with that of the electromagnet.

2. *Experiments on Spirostomum teres.*

Experiment a.—Spirostomum teres is one of the largest of unicellular organisms. The average length of those on which the following experiments were made was about two tenths of a millimeter. Spirostoma from a wild culture were centrifuged to determine their position in a capillary tube in the same way as in the preceding experiments. The majority of the animals were found with anterior ends directed toward the open outer end of the capillary tube. Owing to the animal's extreme contractility and long form, the writer could not obtain such invariable uniformity of results as he did in the case of *Paramecium*. Nevertheless, the proportion thus directing their anterior end with reference to the centrifugal force was sufficiently great to show that the position was not accidental.

Experiment b.—An attempt was made to repeat the iron-ingestion experiments on spirostoma, but without success. The writer mixed iron filings with about 2 c.c. of the "wild" culture containing a dense suspension of spirostoma in the way that is already described. Within five minutes, all of them broke into pieces beginning at the anterior end and continuing to the posterior, even though none of them ingested iron. Here we see the great injury of iron to the animals; so that the iron ingested by paramecia might not be "harmless" to these animals or at least it might produce some chemical effect on them.

III. The Pressure Theory and its Criticism.

Jensen, having been dissatisfied by Verworn's mechanical theory, proposed the "pressure theory" of geotropism (11, pp. 462–464). According to Jensen, the geotropic orientation of paramecia is determined by the difference in hydrostatic pressure between the upper and lower surfaces of the animal. In the negative geotropism, therefore, the animal moves from the place of high pressure to that of low; in positive geotropism, the animal moves from the place of low pressure to that of high (11, pp. 462–463). Davenport first criticized this theory of Jensen's, and suggested a third theory (2, pp. 122–123). Jennings (9, pp. 473–477) and Lyon (16, pp. xv–xvi) independently disproved Jensen's theory from a physical point of view.

Jennings calculated that "the difference in pressure between the two sides of the organisms is only 1/1,000,000 of the pressure acting everywhere on the surface. Furthermore, Jensen showed that the reaction still occurs when the atmospheric pressure is more than doubled; the effective difference in pressure would be less than 1/2,000,000 of the general pressure" (9, pp. 475–

476). Lyon's calculation was practically the same as Jennings, *i. e.*, 1/1,040,000 (16, p. xv). Since it is well known that the "threshold differential required for the perception of differences in pressure" in man is "about 1/10," both investigators cannot believe that a differential of 1/2,000,000 or 1/1,040,000 is perceptible by paramecia. With regard to the difference in pressure between the anterior and the posterior ends, Jennings thinks that the posterior end being only slightly sensitive to a touch to which the "anterior end reacts violently," "an increase of pressure on the posterior end" "would cause no reaction whatever" (17, p. 476). Neither Jennings nor Lyon calculated the difference in pressure between the anterior and posterior ends of the organism, but this is easily made as follows: The atmospheric pressure being 10,000 mm. of water, and the average length of the paramecia which the writer experimented on about 0.2 mm., the writer calculates the difference in pressure between the anterior and posterior ends of the organisms to be about 1/50,000 of one atmosphere. If "the reaction still occurs when the atmospheric pressure is more than doubled," as Jensen showed, "the effective difference in pressure would then be" about 1/100,000 of one atmosphere. It is hardly conceivable that the difference of either 1/50,000 or 1/100,000 of one atmosphere is perceptible to paramecia. Moreover, Lyon's experiments have shown that "geotropism is intensified in a centrifuge tube which is open away from the axis and in which, therefore, no increase of pressure above atmospheric is possible" (17, p. 431). On the basis of such various evidence, we ought to abandon the pressure theory of geotropism in *Paramecium*.

IV. The Resistance Theory.

A third theory of geotropism, proposed by Davenport, is based on the assumption that the animal "experiences greater resistance (friction + weight) in going upwards even to the slightest extent than in going downward (friction − weight)" (2, p. 122). Lyon calls this the "resistance or weight theory" (17, p. 426). This theory of Davenport was once accepted by Jennings (9, pp. 477–480), though now he rather favors Lyon's theory (10, p. 77) which we shall consider later.

If the weight of organisms or their resistance to the pull of gravity is the only cause of geotropism, negatively geotropic animals should become positively geotropic, and positively geotropic animals should become negatively geotropic in solutions of greater specific gravity than their own, as Platt points out (20, p. 31). Platt at the suggestion of Davenport attempted in vain to solve this problem, because she could not find "paramecia which showed decided geotropic reaction" (20, p. 32). The first approach to this problem was to determine the specific gravity of the *Paramecium.* Jensen first reported it as 1.25 (12, p. 544). He killed *Paramecium aurelia* in solutions of potassium carbonate (12, p. 543) and determined their specific gravity when they were suspended in a solution of known specific gravity. But as Platt has shown (20, p. 34), the specific gravity of the animal when so treated was certainly increased in the solution to some extent. The value of the specific gravity of paramecia obtained by Jensen, therefore, is hardly worth serious consideration. Platt then tried to determine the specific gravity by killing the animals in water to which was "added a few drops of 0.5 per cent. acetic acid." She "also killed paramecia by the fumes of osmic acid." Then she placed the killed paramecia "in a gum-arabic solution of 1.018 specific gravity," whereupon they "remained long suspended in the solution" (20, pp. 35–36). So she thinks that the specific gravity of paramecia so treated "is about 1.017" (20, p. 38). But Platt's method was no better than Jensen's. As she observed that the "reagents caused the paramecia to change their shape somewhat in dying, by becoming wide and shorter," we are not justified in assuming that the specific gravity of the dead animals remained the same. "On the death of the cell," according to Lillie, "there follows a marked and permanent increase in the general permeability, and this change is always associated with a permanent fall in the potential difference between surface and interior" (14, p. 196). Furthermore, as Lyon rightly points out, a viscous gum-arabic solution is likely to hold suspended for a long time even bodies of considerably different density "unless a greater force than gravity be used to separate them from the solution" (17, p. 427). We can therefore place little value on either Jensen's or Platt's results.

Lyon then adopted a method "by which the density of small organisms could be found without any injury or change in them." He "made use of the hæmatocrit attachment of the centrifuge by which very high velocities" (from 10,000 revolutions to 12,000 per minute) "of rotation and consequent high centrifugal force are secured. At the bottom of a *small*[1] tube was placed *a little*[1] gum-arabic solution of known density; above this a *few*[1] drops of water containing paramecia. The tube was then centrifuged about one minute." He thus found that "an average density of paramecia is about 1,048 or 1,049" (17, p. 427).

1. *Experiments on Paramecium caudatum.*

In the results of Jensen, Platt, and Lyon, however, the difference of density is so great that the present writer thought it well to repeat Dr. Lyon's experiments, under his direction, with some modifications. The writer prepared a gum-arabic solution using distilled water. The acid of the solution was carefully neutralized with sodium carbonate. But he regrets that he forgot to dialyze it. He made a series of 40 solutions of different densities from 1.02 to 1.05 in Naples jars. The specific gravity of each was carefully determined by means of a finely graded hydrometer. After some trials the solutions above and below the densities given in the following table were found unnecessary. Then he prepared many small tubes about 12.5 cm. long and 0.4 cm. in diameter, one end of which was sealed. In each trial, two of those tubes were filled about 11 cm. high with gum-arabic solutions of known specific gravity, *e. g.*, the one 1.02 and the other 1.021. This pair was centrifuged to drive the air out. Then on the top of each solution in the two tubes, *one* drop of the culture containing dense paramecia was added by a very small pointed pipette. The tubes so prepared were again centrifuged for one minute with a speed of about 1,300 revolutions per minute. The distribution of the animals in both tubes was then carefully compared. All 40 different solutions having been thus tested, the nearest possible density of the animals was obtained:

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[1] Italics not in the original.

I.	Tube 1.	Density of gum-ar. sol. 1.034,	1,300 revolutions; a few go to bottom.
	Tube 2.	Density of gum-ar. sol. 1.035,	1,300 revolutions; very few go to bottom.
II.	Tube 1.	Density of gum-ar. sol. 1.036,	1,300 revolutions; 2 or 3 go to bottom.
	Tube 2.	Density of gum-ar. sol. 1.037,	1,300 revolutions; none go to bottom.
III.	Tube 1.	Density of gum-ar. sol. 1.038,	1,300 revolutions; a few go to lower part than middle.
	Tube 2.	Density of gum-ar. sol. 1.039,	1,300 revolutions; some go to middle.
IV.	Tube 1.	Density of gum-ar. sol. 1.030,	1,300 revolutions; many go to bottom.
	Tube 2.	Density of gum-ar. sol. 1.043,	1,300 revolutions; many stay at top.

The writer found thus that the density of the *Paramecium caudatum* is about 1.036 or 1.037. To make sure of the results, three different series of the same experiments were made with different cultures in each series. From two cultures, practically the same results were obtained as the above, but it was found in one culture that the animals when centrifuged were so weak that they were all broken into pieces on the bottom.

In comparison with Lyon's 1.048 or 1.049, we find a difference of about 0.012. Although the difference does not seem very great, it is great for such small organisms. Accordingly at the suggestion of Dr. Knowlton and Dr. Lyon, the writer tested the hydrometer which he used for the measurement of the density of the gum-arabic solutions, by means of a density bottle with Dr. Lyon's help. It was found that the weighing difference between the hydrometer and the bottle was about 0.003. If the test was correct, we may add this difference, 0.003, to 1.036 or 1.037, which makes the density 1.039 or 1.040. If so, the difference between Lyon's 1.048 or 1.049 and the writer's 1.039 or 1.04 becomes a little less.

The only possible source of error in Lyon's experiments is that he may have placed too much "water containing paramecia" above "*a little* gum-arabic solution" in the "*small*" and short tube which he adopted for the hæmatocrit attachment. Under such circumstances, possibly the animals when centrifuged carried some water with them into the gum solution and lowered the known density of the latter. Nevertheless, there may exist some difference in the specific gravity of the animals in different cultures, as seems to be indicated by the experiments described. This must be borne in mind.

Then the writer prepared a series of 10 tubes about 12.5 cm. long and 0.4 cm. in diameter, in each of which was a definite gum-arabic solution of known density—from 1.034 to 1.044—thoroughly mixed with about two drops or slightly less of the culture containing dense paramecia, filling each up to 12 cm. Three drops of paraffine oil were placed on top of the solution "to exclude oxygen and a possible consequent chemotropic gathering." The writer's observations upon the paramecia so treated agreed with Lyon's in all respects. His words may, therefore, be quoted: "Invariably in all solutions, whether of equal, greater, or less density than the animals, the latter slowly rose and formed a dense ring near the top. The response was slow, as the velocity of swimming in such viscous solutions is extremely small. Two or three hours, or over night, was often necessary. But often the rising ring of animals could be seen in twenty minutes or less. It was typical geotropism very much slowed" (17, p. 428).

The animals so treated lived 5 days in all the solutions above mentioned, though the number of the animals had noticeably decreased at that time. A week after, all the animals in the solutions of densities 1.044 to 1.037 had died, though a few animals in the solutions from 1.036 to 1.034 lived for 13 days. It is rather interesting therefore to note that the animals in the solutions of the greater densities than their own died earlier than the animals in the solutions of the lesser densities. This may not be simply due to density, but density may be at least one of the causes of death of the animals.

To avoid the criticism that the specific gravity of the animals may have been increased by a prolonged stay in such solutions, the writer carefully tested the matter, as Lyon did. After several tests, he found that the animals had increased their density on an average between 0.002 and 0.003 after 13 hours in the solutions. This was determined by centrifuging tubes in which the animals were suspended in solutions of known densities.

The distribution shown by the animals was then compared with the distribution shown in the original determinations of their specific gravity. If the animals in the solution of 1.037 increased their density about 0.003 after 13 hours, they should be distributed in the tube when centrifuged at this time as they had formerly been in the solution of 1.034. The results of Lyon and those of the writer agree fairly with one another in this respect (17, p. 428).

From the results of all the above experiments, we have found that the weight or resistance theory of geotropism is untenable. We have found that no animals which are negatively geotropic become positively geotropic in gum-arabic solutions of greater specific gravity than their own. Thus Lyon's results in all respects are fully confirmed except as regards the exact value of the specific gravity of the animals.

2. *Experiments on Spirostomum teres.*

The same experiments as the above were made with Spirostoma in the same ways. The density of the animals was found:

I.	Tube 1.	Density of gum-ar. sol. 1.022,	1,000 revolutions; some go to bottom.
	Tube 2.	Density of gum-ar. sol. 1.023,	1,000 revolutions; a few go to bottom.
II.	Tube 1.	Density of gum-ar. sol. 1.024,	1,000 revolutions; 4 go to bottom.
	Tube 2.	Density of gum-ar. sol. 1.025,	1,000 revolutions; none go to bottom.
III.	Tube 1.	Density of gum-ar. sol. 1.026,	1,000 revolutions; a few go to lower part.
	Tube 2.	Density of gum-ar. sol. 1.027,	1,000 revolutions; a few go to middle.
IV.	Tube 1.	Density of gum-ar. sol. 1.019,	1,000 revolutions; most go to bottom.
	Tube 2.	Density of gum-ar. sol. 1.030,	1,000 revolutions; most stay near top.

These experiments show that the specific gravity of *Spirostomum teres* lies between 1.024 and 1.025, or very near to 1.025. If we add 0.003 to the 1.025, as we did before, it becomes about 1.028.

Platt obtained a specific gravity of spirostoma of about 1.017 (20, p. 34), which varies by about 0.008 from 1.025. As we have already pointed out, Platt's method was not as accurate for obtaining the density of the animals as Lyon's. We may therefore consider our results to be the more accurate.

Then nine tubes about 12.5 cm. long and 0.4 cm. in diameter were prepared. They were filled up to about 12 cm. with the solutions of known density—from 1.24 to 1.032—thoroughly mixed with about two drops of the "wild culture" containing dense spirostoma, free from sediment. Three drops of paraffine oil were placed above the solution in each tube.

The negative geotropism of spirostoma is not so marked as usually supposed, and their movements under these conditions were much slower than those of the paramecia in the preceding experiments. About two hours after placing in the solutions of the same density as their own a few rose up to about the middle part of the tubes; a still larger proportion so rose in the solutions of greater densities than their own, but in the solution of 1.024 all were on the bottom, while in that of 1.025 a few rose from the bottom of the tube to the height of 2.5 cm. This suggests that the buoyancy in the solutions of greater densities than their own helped the animals to rise. But about 13 hours later, nearly all the animals in the solutions rose up to near the top. Two weeks later some spirostoma were living in all the tubes except one of 1.027 which was broken 4 days after treatment. At that time the writer had to leave Woods Hole. Meanwhile the number of the animals considerably decreased, but in some tubes a few individuals divided.

V. THE STATOCYST THEORY AND ITS CRITICISM.

A fourth theory of geotropism proposed by Lyon is based on the assumption that the *Paramecium* contains protoplasmic materials of different specific gravity. "For internal stimulation the relation of the parts of the cell to each other must be changed in some way by grvaity. Stresses or pulls which occur when the organism is in one position with respect to the vertical, must be changed in another position" (17, p. 429). This is called the "statocyst theory" of geotropism in the *Paramecium*. It is worth while to mention, with reference to the general theory of geotropism in animals, that Loeb in 1897 (15, pp. 446–449) had already suggested a similar theory.

Lyon furnishes evidence (though not conclusive) for his theory as follows: "The animals were strongly centrifuged for several minutes in the hæmatocrit attachment. Microscopic examination showed that certain dark granules originally distributed were now aggregated in one end, usually the anterior. It is thus seen that differences in specific gravity exist in the protoplasm of this animal" (17, p. 430).

The writer tried, by means of the centrifuge, to test Lyon's results, but must confess that he could obtain nothing definite. But he thinks now that he ought to have adopted some chemical methods (stains) which might possibly show better results. But since all theories based on the results of external stimulation have been shown above to be fallacious, this theory of Lyon's seems from a physico-chemical point of view to be the most possible and the most reasonable. If this theory is tenable, the matter of the heaviness of either anterior or posterior end is of very little significance in such a unicellular organism as *Paramecium*, because even the lighter end of the organism may possibly contain protoplasmic materials of a specific gravity greater than that of this region taken as a whole.

VI. Conclusions.

So far as the results of our experiments and analyses are concerned, the geotropism of paramecia is due chiefly, if not entirely, to the internal conditions of the organisms rather than to external ones. The results of our experiments have shown that the anterior ends of the animals are heavier than their posterior ends. This fact gives no direct clue to our problem, but furnishes strong evidence against the mechanical, that is, external, theory. On the other hand, if we suppose with Harper that the posterior ends of the animals are "heavier" than their anterior ends, that the "pull of gravity" "is too weak to stimulate the organisms," and that the consequent negative geotropism is due to the orientation of the animals in a "purely mechanical" way, we have no explanation for the lack of uniformity in response to the same stimulus under varying conditions. For instance, when given a "shock," the normal paramecia which form a ring at the top of a tube become positively geotropic and swim downward, even though the "shock" lasts only a moment. Moreover, as Sosnowski (23, p. 134) and Moore (19, pp. 239–241) have shown, the animals are negatively geotropic at 2° C., while they are positively geotropic at 30° C. As we have already pointed out, certain spiders whose posterior region is much heavier than the anterior, nevertheless orient themselves, when at rest with their head down. Therefore we conclude that without consideration of the internal factors, we have difficulty in explaining the facts, even though we do not absolutely reject the external factors.

A possible method which the writer intends to try later would consist in the following: Immediately after centrifuging a capillary tube containing paramecia, and in which the latter cannot turn around, we may stain them with some dyes and determine the effects of the centrifugal force upon the protoplasmic materials of the organisms. The application of this or similar methods is of the utmost importance for the problem of geotropism not only in paramecia but in animals in general.

VII. Summary.

1. *Paramecium caudatum* and *Spirostomum teres* assume a position with their anterior ends directed away from the axis of the centrifuge. Their anterior ends, therefore, must be heavier than their posterior ends. If so, the negatively geotropic orientation is an active process and the mechanical theory cannot be held.

2. It is not conceivable that the animal could be sensitive to slight differences in pressure such as we have computed must exist between the two sides or the two ends of its body. Therefore the pressure theory is not tenable.

3. *Paramecium* and *Spirostomum* in the gum-arabic solutions of greater, equal, and less specific gravity than their own are still negatively geotropic. Therefore the resistance or weight theory is not correct.

4. The specific gravity of living *Paramecium caudatum* is about 1.037 or 1.037 ± 0.003.

5. The specific gravity of living *Spirostomum teres* is about 1.025 or 1.025 ± 0.003.

6. The statocyst theory of geotropism is the most tenable.

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PURE LINE INHERITANCE AND PARTHENOGENESIS.

H. E. EWING.

Introduction.

Ever since Johanssen, by his epoch-making researches with self-fertilized strains of beans, established his pure line conception of heredity, other investigators have been extending this conception so as to have it include many forms which do not reproduce sexually. In fact some of these investigators hold that all selections made within isolated strains of asexually reproducing plants and animals will fail to alter in the least the fraternal mean of succeeding generations.

Some careful work already has been done with lower organisms, which shows that Johanssen's pure line conception holds true for forms reproducing by fission or budding. Up to the present time no extensive work has been done with parthenogenetic animals. In fact the only paper which I have found dealing with selections among parthenogenetic forms is one by Kelly. Kelly worked with an aphid, *Aphis rumicis*, but did not carry his selections beyond two generations. However, he came to the conclusion that, "the progeny does not inherit the somatic idiosyncrasies of the parent, but does inherit from the underlying germ plasm common to all."

During the winter of 1910–'11, I began a series of experiments along pure lines with the cabbage aphis, *Aphis brassicæ* Linn., but while moving about from one part of the country to another I confined the individuals too closely in their breeding cells with the unfortunate result that they all died.

Again during the summer of 1912 I took up similar experiments with our common black cherry aphis, *Myzus cerasi* Fab., but before I obtained a sufficient number of generations to make the results reliable, cold weather set in causing the sexual forms to appear, thus spoiling my results.

While working this last winter with the European grain aphis, *Aphis avenæ* Fab., I noticed its great variations, and since the species was already reproducing parthenogenetically under laboratory conditions, I decided to take up for the third time heredity work with a parthenogenetically reproducing insect. This time my efforts have been rewarded with some measure of success. Most of the results obtained up to the present are here submitted.

Life History and Habits of Aphis avenæ Fab.

Before submitting the results of these experiments, and entering upon a discussion of them, I shall review briefly the life history and habits of the species under consideration.

As is true of aphids in general, this species propagates itself chiefly by means of parthenogenesis, but under certain conditions (probably due chiefly to climatic changes) the true sexes appear, mating occurs, eggs are deposited, and the parents die. In the case of this species, according to Pergande, the true sexes do not appear annually under out-of-doors conditions; but the asexual females live over the first winter, the true sexes being produced the next fall. Thus two years are required to complete the cycle from egg stage to egg stage.

Aphis avenæ lives on a large number of plants, but is especially injurious to small grains and grasses. It will frequently winter over on apple, pear, plum, or other deciduous trees. The eggs that have passed the winter out of doors usually hatch about the middle of March in most of the eastern states. It takes these young aphids about a month to reach maturity; when they are called the stem-mothers. The offspring of these stem-mothers are all asexual females, and produce parthenogenetically, for several or many generations, other asexual individuals. These asexual females are of two classes; winged and wingless. Although the winged or wingless condition of these two classes constitute the chief ground for their differentiation there are other differences also, but generally these are not very important.

During the warmer part of the season the asexual females reproduce very rapidly, and the time required for the complete life cycle may not be more than six days. During the colder parts of the year two weeks or even more may be required for the completion of the life cycle. The asexual individuals vary greatly in their period of longevity and in their fecundity. Some will die in a few days after reaching maturity, and will leave few or no offspring. Others may live for two weeks or more, and leave at least a score of offspring.

The young when born are quite similar to the adults except in size. They at once begin to feed, and after molting three or four times they reach maturity. Although the immature individuals are quite content to settle on the same stalk next to their mother, and to remain there throughout their period of growth, when they reach maturity, especially if they are winged, they are restless, and generally leave the plant upon which they have been reared.

VARIATIONS.

This aphis is quite variable in several respects. Pergande, who has made a special study of the species, states: "I have found a certain range of variation in the comparative length in the joints of the antennæ, as well as in the nectaries of the progeny of the apple louse [*Aphis avenæ*], the extreme forms of which may easily induce superficial students to consider them as distinct. Large series, however, of the various forms, more or less due to the season or abundance of food, have convinced me that all of them belong to but one species."

I cannot agree with Pergande in attributing these variations entirely to the season or the food supply. For ten generations I have reared these aphids exclusively on young wheat shoots, and I find that most of these variations obtain even in their extremes when all individuals are reared on the same diet. However, the food supply certainly does affect some of the variations, but most of them originate without a change in the amount or nature of food taken. What is more significant, I find these variations existing among offspring of a single female reared under identical conditions and even find the antenna on one side differing from the antenna on the other side in the same individual. In regard to this latter point, however, I find that for a large majority of the individuals examined the antennæ on the two sides of the same individual are the same or nearly so.

After studying the different characters which were available I finally decided to use those of the antennæ. The variations in the relative lengths of the third and fourth segments were observed and employed. It is interesting to note that these characters are the same as those I selected two years ago when working with *Aphis brassicæ* Linn.; they are also the same as those used by Kelly in his work with *Aphis rumicis*.

METHODS.

In order to obtain as nearly as possible uniform conditions for these experiments, I reared all of the aphids on young wheat plants. These wheat shoots were never allowed to get to be more than ten or fifteen days old, and I kept them nipped back to a height of not more than three or four inches. New plants were reared from seed for each generation of plant lice. All of the plants were kept upon a single large table in the middle of the laboratory, so that changes in heat or moisture would affect all individuals of each fraternity in much the same way.

The wheat plants used were grown in small flower pots, a single plant being raised in each pot. Soon after the aphids were born they were separated, and each nymph was placed on a single wheat plant growing in its own pot. This aphid was confined by placing a small lamp chimney, covered at the top with surgeon's gauze, over the wheat plant. This would permit ready observation of the developing aphid, and would also permit the transpiration of the wheat plant. Each individual was given a label which showed the generation to which it belonged, and also its individual number in this generation. Thus, $F_7 14$ would mean individual number fourteen of the seventh filial generation.

When it came to taking measurements of the third and fourth segments of the antennæ in order to record the same and to select individuals for carrying on the strain I encountered several difficulties. At first I tried to measure these in the live adults by weighing down the insect with a cover-glass, but this would not enable me to make accurate measurements. After trying several other methods I finally decided to let each adult remain on its own wheat plant, and produce her progeny; after which she was killed, and the head, with the antennæ, was severed from the body, cleared in Gage's mixture, and mounted in balsam on a microscope slide. This method I found to be the only satisfactory one, for two reasons. First, measurements of the segments of the antennæ could be made accurately; and second, the fecundity of the individuals could be tested before the selection was made, a very important factor, since it frequently happens that some adults have very few or no offspring.

Having made the measurements of the antennal segments, and obtained the index for the same, I selected from each fraternity the individual or individuals desired. These were usually those whose antennal index was either the lowest or the highest in the fraternity, provided they had left a sufficient number of offspring. Now the offspring of the individuals selected were separated and placed on individual plants; those of the other individuals of the fraternity were discarded.

In making the measurements of the segments of the antennæ a mechanical eye micrometer was used. Both antennæ were measured, and an average obtained from the two measurements in order to get the index for the individual. From these indices ratios were obtained, segment four being taken as unity.

Having obtained the ratio of the length of segment three in each case to the length of segment four, I have plotted (Fig. 1) the results of the selections for ten generations. For the first six generations random selections were made in order to be sure of a pure strain and to determine the average mean index for this strain. The mean thus obtained gave a ratio of 1.80:1, that is, segment three of the antennæ was found to be one and eight tenths times as long as segment four. This ratio established for the pure line is represented by the heavy horizontal line running through about the middle of the plot in Fig. 1.

RESULTS.

On April 9 the first individual was isolated, a wingless asexual female, and she became the mother from which all the succeeding individuals were descended. This stem mother gave birth to twenty-nine offspring. She was now killed and her antennal index taken. It was found to be 1.77:1. From the twenty-nine offspring I selected one at random, with which to carry on the strain. The individual selected was F_13 (that is, individual number three of the first filial generation). This individual gave birth to ten young. Her antennal index proved to be 1.77:1, the same as that of her mother. From the F_2 individuals I selected at random again an individual which became the mother of six offspring. Her index was, as later obtained, 1.67:1, while that for the mean of her offspring was 1.77:1.

For the next three generations selections were made at random, a prolific or healthy looking individual being taken. The measurements of the antennal segments of all the individuals were taken, and from these the fraternal means for the second, third, fourth, and fifth generations obtained. For the first filial generation I obtained the index of the single individual used to carry on the strain. Using this index and that of the stem mother I obtained a curve for the first five generations. This I have plotted (see Fig. 1). It will be noted here that in the case of the selections of individuals representing extreme variations, as was done in the second and third filial generations, that the mean of their offspring came back to or almost to that of the strain.

In the fourth generation an individual was selected which had an antennal formula near that for the mean of its fraternity, and exactly the same as that obtained for the strain; yet its offspring gave a mean much higher than that of any individual belonging to the fraternity from which the mother was taken! (See Fig. 1.) More than this, the mean of this fraternity was higher than the index for any individual of any fraternity previously obtained. From the individuals of the F_5 generation one was obtained with a low index. It gave a brood of normal individuals.

It was from among the individuals of the F_6 generation that I began making my selections in an attempt to obtain two strains, one with a high antennal formula, the other with a low antennal formula, from this isolated pure line of parthenogenetically reproducing plant lice. F_65, with a formula of 1.88:1, was selected for obtaining a strain with a high index; F_63, formula 1.66:1, was taken for obtaining a strain with a low index. F_65

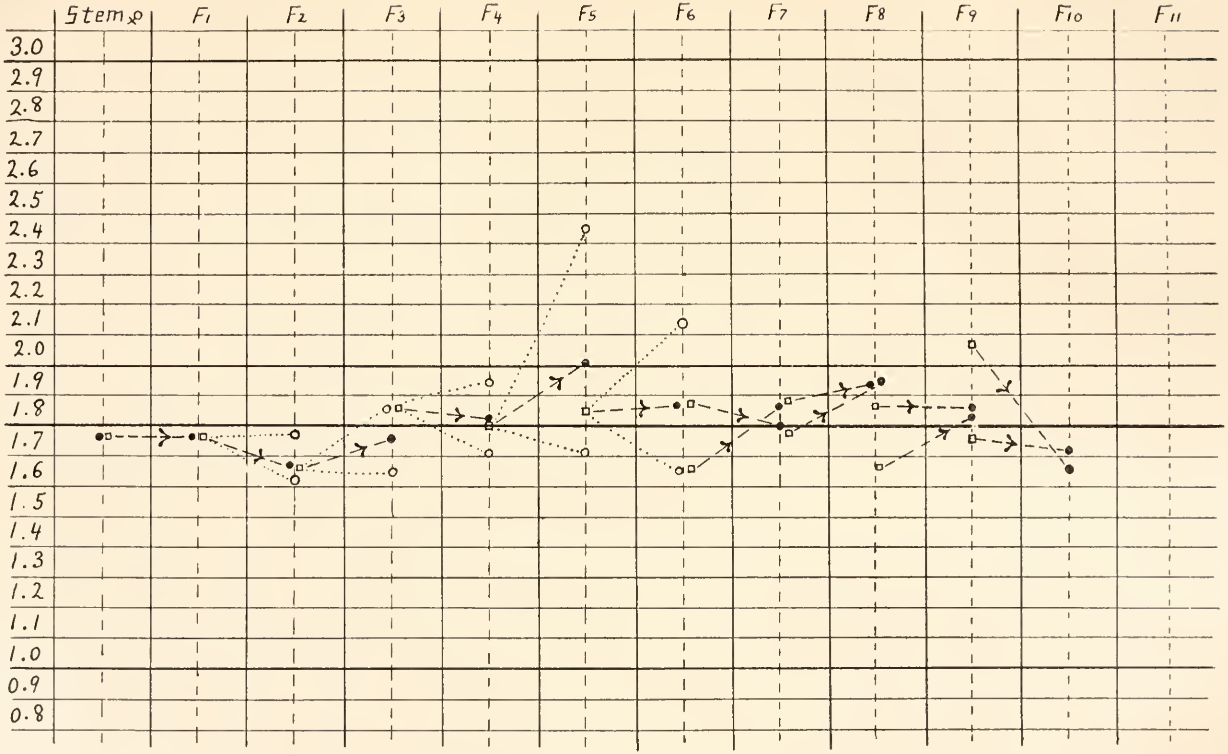

FIG. 1. Diagram showing the plotted results of selections within a pure line of *Aphis avenæ* Fab. Each black circular spot represents the position of a fraternal mean; the open circles represent the positions of the extreme variants of a fraternity; each square represents the position of the individual selected for carrying on the strain or pseudostrains. The arrows indicate the course taken by the strain as it passes from the selected individual to the mean of its offspring (the fraternity of the next generation). The heavy line at 1.8 represents the position of the mean of the strain, and may be called the mean-of-the-strain base line.

TABLE OF MEASUREMENTS AND RATIOS.

Generation and No. of Individual.	Wings.	Left Antenna.		Right Antenna.		Average for Both Antennæ.		Rati
		3d Jt.	4th Jt.	3d Jt.	4th Jt.	3d Jt.	4th Jt.	
Stem ♀	—	3.20	1.80	Lost	......	3.20	1.80	1.77:1
F$_1$3	—	3.70	1.80	3.70	2.25	3.70	2.025	1.82:1
F$_2$1	—	3.40	1.95	3.20	2.00	3.30	1.975	1.67:1
F$_2$3	—	3.35	1.80	3.15	1.90	3.25	1.85	1.78:1
F$_2$8	—	3.50	2.15	Lost	......	3.50	2.15	1.63:1
F$_3$1	—	2.95	1.50	2.90	1.65	2.925	1.575	1.86:1
F$_3$3	—	2.70	1.65	2.50	1.50	2.60	1.575	1.65:1
F$_3$5	—	3.05	1.60	2.85	1.70	2.95	1.65	1.79:1
F$_4$1	—	2.40	1.40	Broken off		2.40	1.40	1.71:1
F$_4$3	—	2.35	1.25	2.15	1.25	2.25	1.25	1.80:1
F$_4$6	—	Broken off		2.45	1.25	2.45	1.25	1.96:1
F$_5$1	—	2.70	1.10	2.70	1.10	2.70	1.10	2.45:1
F$_5$2	—	2.65	1.10	2.15	1.00	2.40	1.05	2.28:1
F$_5$3	—	3.15	1.75	3.20	1.85	3.175	1.80	1.76:1
F$_5$6	—	2.80	1.50	2.80	1.75	2.80	1.625	1.72:1
F$_5$5	—	3.75	1.80	3.30	1.65	3.525	1.725	2.04:1
F$_5$10	—	3.70	1.80	3.70	2.20	3.70	2.00	1.85:1
F$_6$1	—	2.75	1.50	2.75	1.50	2.75	1.50	1.83:1
F$_6$3	—	3.10	1.85	3.15	1.90	3.125	1.875	1.66:1
F$_6$5	—	3.05	1.65	3.25	1.70	3.15	1.675	1.88:1
F$_6$6	—	3.15	1.70	Broken off		3.15	1.70	1.85:1
F$_6$2	+	Broken off		3.60	1.80	3.60	1.80	2.00:1
F$_6$4	+	3.25	1.65	3.70	1.60	3.475	1.625	2.14:1
F$_7$3	—	3.60	2.00	3.65	1.82	3.625	1.91	1.89:1
F$_7$4	—	3.30	1.85	3.40	1.90	3.35	1.875	1.79:1
F$_7$2	—	4.00	2.00	3.80	2.20	3.90	2.10	1.86:1
F$_7$6	—	Lost	......	4.15	2.10	4.15	2.10	1.98:1
F$_7$8	—	3.85	2.25	Lost	......	3.85	2.25	1.71:1
F$_7$11	—	3.70	1.95	Broken off		3.70	1.95	1.90:1
F$_8$1	—	4.60	2.55	4.60	2.40	4.60	2.475	1.86:1
F$_8$12	—	3.55	1.75	3.50	2.10	3.525	1.925	1.83:1
F$_8$2	+	4.70	2.25	4.70	2.40	4.70	2.325	2.02:1
F$_8$3	+	Misplaced		4.95	2.50	4.95	2.50	1.98:1
F$_8$4	+	4.30	2.20	4.70	2.45	4.50	2.325	1.93:1
F$_8$5	+	3.70	1.95	3.95	2.00	3.825	1.975	1.99:1
F$_8$6	+	4.20	2.05	3.75	2.00	3.975	2.02	1.91:1
F$_8$7	+	4.10	2.20	4.00	2.05	4.05	2.125	1.90:1
F$_8$8	+	4.00	2.00	Broken off		4.00	2.00	2.00:1
F$_8$10	+	Broken off		4.75	2.40	4.75	2.40	1.98:1
F$_8$11	+	4.20	2.16	3.85	2.20	4.025	2.18	1.85:1
F$_8$13	—	4.20	2.30	4.15	2.30	4.175	2.30	1.81:1
F$_8$16	—	3.40	1.17	3.40	1.17	3.40	1.17	2.90:1
F$_8$17	—	3.80	2.25	3.75	2.15	3.775	2.20	1.71:1
F$_8$19	—	3.60	2.20	3.60	2.10	3.60	2.15	1.67:1
F$_8$14	+	4.10	2.30	4.10	2.30	4.10	2.30	1.78:1
F$_8$15	+	4.25	2.15	Broken off		4.25	2.15	1.97:1
F$_8$18	+	3.40	1.80	3.20	1.80	3.30	1.80	1.83:1
F$_9$17	+	3.20	1.80	Displaced		3.20	1.80	1.77:1
F$_9$18	+	3.35	1.80	3.05	1.80	3.20	1.80	1.77:1
F$_9$19	+	4.00	2.20	Broken off		4.00	2.20	1.81:1
F$_9$20	+	3.85	1.75	3.75	1.95	3.80	1.85	2.05:1
F$_9$22	+	3.60	2.05	3.60	2.00	3.60	2.025	1.77:1
F$_9$1	+	3.30	2.00	3.35	2.00	3.235	2.00	1.66:1
F$_9$2	+	Broken off		3.35	1.75	3.35	1.75	1.91:1

TABLE OF MEASUREMENTS AND RATIOS (*Continued*).

Generation and No. of Individual.	Wings.	Left Antenna.		Right Antenna.		Average for Both Antennæ.		Ratio.
		3d Jt.	4th Jt.	3d Jt.	4th Jt.	3d Jt.	4th Jt.	
F$_9$3	+	Broken off		Broken off		. . . .	. . . .	. . . .
F$_9$4	+	Broken off		3.40	2.00	3.40	2.00	1.70:1
F$_9$5	+	3.20	1.75	3.35	1.95	3.275	1.85	1.77:1
F$_9$6	+	3.80	1.80	3.30	1.70	3.55	1.75	2.03:1
F$_9$8	+	3.60	1.85	3.35	2.00	3.475	1.925	1.80:1
F$_9$9	−	3.70	1.80	3.80	1.80	3.75	1.80	2.08:1
F$_{10}$1	+	2.90	1.80	3.10	1.75	3.00	1.775	1.70:1
F$_{10}$2	+	Broken off		2.80	1.75	2.80	1.75	1.60:1
F$_{10}$3	+	3.00	1.65	Displaced		3.00	1.65	1.82:1
F$_{10}$4	−	3.15	1.80	3.00	1.85	3.075	1.825	1.63:1
F$_{10}$5	−	2.50	1.50	2.50	1.50	2.50	1.50	1.66:1
F$_{10}$6	−	3.25	1.90	3.30	2.05	3.275	1.975	1.65:1
F$_{10}$7	−	2.50	1.60	2.50	1.60	2.50	1.60	1.56:1
F$_{10}$11	−	2.40	1.40	Broken off		2.40	1.40	1.71:1

gave me five young, only two of which came to maturity and gave an average index of 1.80:1. F$_6$3 gave birth to eight individuals, four of which came to maturity, and gave as a fraternal mean an index of 1.88:1. Although the number of individuals obtained are too small to draw any conclusions, if they were not supported by a repetition of similar results, yet they are very significant, as is shown by the plot in Fig. 1. The curves running from the two extreme individuals selected of the F$_6$ generation, to the fraternal means of the offspring of these two individuals actually cross! In other words, offspring from an individual with a high index had a lower fraternal mean than the offspring from the individual (of the same fraternity as the former) with a low index. Among the F$_7$ individuals two were selected; one, F$_7$3 with an index of 1.89:1, and one, F$_7$4, with an index of 1.79:1. From the former, F$_7$3, eleven young were reared to maturity. They gave a fraternal mean of 1.93:1. This is almost the same formula as was obtained for the mean of the offspring of the latter individual, F$_7$4, which was 1.95:1. Here we get convergence almost to the same point in the offspring of two variants of a fraternity. However, both of these fraternities, that is, of the F$_8$ generation, did not show a reversion toward the mean of the original strain, but a deviation from it. This is what occurred in the case of the F$_5$ generation. In the F$_8$ fraternity, F$_8$1, formula 1.86:1, and F$_8$19, formula 1.67, were selected, each being a descendent of a different individual of F_7 generation. $F_8$1 gave offspring with a mean of 1.85:1, and $F_8$19 offspring with a mean of 1.83:1. The former converged slightly toward the mean base line of the original strain, the latter reverted not only to it but beyond it, so that the means of the two fraternities almost meet. From these fraternities $F_9$9, formula 2.08:1, and $F_9$17, formula 1.77:1, were taken, each again being selected from the different pseudostrain. Although the former had an antennal formula very high, 2.08:1, in fact higher than that of any individual selected during the whole experiment, yet its seven offspring gave the remarkably low fraternal mean of 1.66:1. Thus reversion in this case was not to the mean of the original strain but much beyond it, as has been noted before in the case of selections from the F_6 and F_8 generations. From $F_9$17, only a single individual was obtained which gave a formula of 1.71:1, a slight deviation from the mean base line.

Conclusions.

The following conclusions can be drawn from the results obtained. Selections from among extreme variants do not alter the mean as obtained for the strain without selection. The fraternal mean of any generation may show a great fluctuation from the mean of the strain, but this fluctuation is not transmitted to following generations. The offspring of an extreme variant may show not a reversion to the mean of the line or strain, but a reversion which swings pendulum-like much beyond this mean, only to be brought back to its former side of the mean-of-the-strain base line in the next generation. This phenomenon I call oscillating reversion. Great deviations from the mean of the strain occur among fraternities which have been produced by individuals whose formula is almost the same as that of the mean of the strain. This great divergence is due to some underlying cause not yet determined, and is independent of the nature or amount of food.

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THE AXIAL GRADIENT IN CILIATE INFUSORIA.

C. M. CHILD.

FROM THE HULL ZOÖLOGICAL LABORATORY, UNIVERSITY OF CHICAGO.

The discovery of axial gradients in rate of metabolic reactions in *Planaria* (Child '12, '13*a*, '13*c*) and various other forms together with a considerable body of evidence, mostly as yet unpublished, which points to a close relation between such axial gradients and polarity and symmetry suggested the desirability of determining whether there is any indication of the existence of such axial gradients in those protozoa which possess a definite morphological polarity. If an axial gradient in rate of metabolic reactions is the basis of organic polarity or is in any way associated with it we must expect to find such a gradient in single cells where a definite stable polarity exists as well as in multicellular axiate organisms. The results obtained with various ciliates have fully justified this expectation. In all the forms tested thus far evidence for the existence of an axial gradient has been found.

I. METHODS.

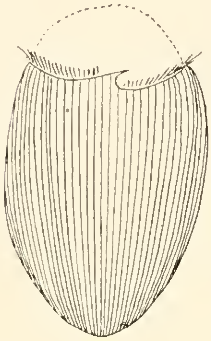
In these experiments the method which I have called the direct susceptibility or resistance method (Child, '13*b*) was used, with KCN as the reagent in most cases. This method is based upon the fact determined by extensive experimentation, that in concentrations of the cyanides and various anesthetics which kill within at most a few hours, individuals or regions with a higher rate of metabolism or at least of the oxidation processes are more susceptible and die earlier than those with a lower rate.

In the experiments with ciliates concentrations were found which did not kill the animals at once but permitted life to continue for a period ranging from a few moments up to several hours according to the concentration. The purpose in thus limiting the concentrations used is first to avoid killing the animals too rapidly, in which case the death and disintegration of all parts may be so nearly simultaneous that a gradient if present will be scarcely or not at all apparent. If on the other hand the concentration is too low, the results may be complicated by acclimation, the individuals or regions of highest rate showing in general the greatest capacity for acclimation. The proper concentrations for each species must of course be determined by experiment.

II. HETEROTRICHA.

The species *Stentor cœruleus* has been the chief experimental material from this group. In KCN the animals do not become elongated and attach themselves but contract to a rounded form and swim continuously backward by means of the cilia. In KCN 0.002 *m*. at a water-temperature of 25°–26° C. all animals (several hundred in each experiment) remain alive and intact and swim continuously during the first hour. At the end of this time a very few individuals show the first stages of disintegration. From this time on death and disintegration occur until at the end of the second hour all are dead. At lower temperatures the survival time is longer.

The usual course of death and disintegration is indicated in Figs. 1–5. In each figure the disintegrated portion is indicated merely by a dotted outline. While the animal is swimming about the peristome region or some part of it, often a region near the mouth first, begins to swell and bulge. This change spreads over the whole peristome within a few seconds or in many individuals is practically simultaneous in all regions of the peristome (Fig. 1). This change is accompanied by complete disappearance of the ectoplasmic striations and other structure in the region involved and the entoplasm slowly swells and spreads out. Within two or three seconds after the first indications of change a halo of blue pigment appears in the water about the disintegrated region which has now lost most of its blue color. After a short pause varying in length in different individuals from twenty or thirty seconds to two or three minutes the disintegration extends beyond the peristomal cilia and begins its course toward the aboral end of the body (Fig. 2), obliterating the morphological structure of the cytoplasm as it proceeds. The boundary between the disintegrated and intact region is always sharp and clear and the course of disintegration can be followed without the slightest difficulty. The change at any given level is almost instantaneous: the structure seems simply to dissolve, the cytoplasm swells and the halo of blue pigment appears in the water while the disintegrated part loses most of its color. The peristomal cilia continue to beat until the instant when the boundary of the spreading areas of disintegration reaches them when they suddenly stop in most cases, although occasionally they may beat for a few seconds more, but in all cases they disintegrate very soon. As soon as the



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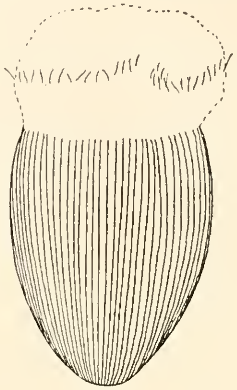

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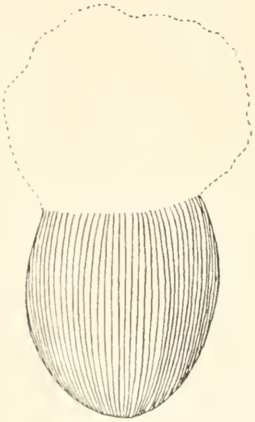

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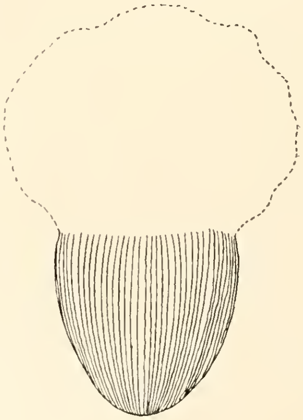

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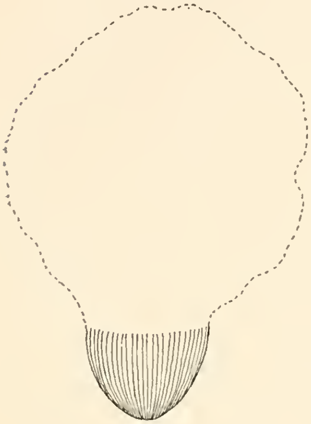
peristomal cilia cease their movement the body ceases to revolve in the water, but the smaller cilia covering the body continue to beat until the disintegration wave reaches them. From the peristome region the process of disintegration spreads downward over the body as indicated in Figs. 2–5, the aboral end being the last region to lose its structure. The disintegration of the aboral half is in practically all cases much more rapid than that of the oral half; very commonly the disintegration of the oral half requires two to four minutes, that of the aboral half one to two minutes, *i. e.*, the differences in susceptibility beween different levels are much greater in the anterior than in the posterior half. Moreover, the anterior half and particularly the peristome region lose their pigment more completely and swell and spread out to a greater extent in disintegration than the posterior half. Finally the animal is represented by a shapeless mass of protoplasm in which the meganucleus remains intact for a long time and about which a halo of blue pigment appears. With the concentration and temperature above mentioned the whole process of death and disintegration requires from two to six minutes, the rate of advance of the disintegration varying in different individuals and in some cases in different regions of the body.



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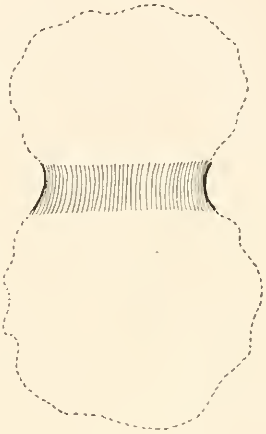

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The striking features of the change are its very definite course, the distinct boundary between disintegrated and intact regions at all times and the uniformity with which the change advances at all points of the circumference of the body. Occasionally, however, modifications of the process appear. In a small percentage of the animals used for the experiment, not more than one or two per cent., the advance of disintegration ceases for a time when it has reached about the stage represented in Fig. 3, *i. e.*, when about the oral half is disintegrated. Then after one or two minutes the disintegration of the aboral half occurs almost simultaneously in all parts. In one individual observed (Fig. 6) the disintegration of the oral half occurred in the usual manner, then there was a pause of about one minute and then the disintegration of the aboral half began from the aboral end. Fig. 6 shows this individual a few seconds before disintegration was complete. A narrow band of intact body wall on which the cilia were seen to be still moving separates the two areas of disintegration. During the next few seconds the aboral area advanced over this band and completed the process. This is the only case among thousands observed in which disintegration proceeded from the aboral end instead of toward it.

The length of life in the reagent before disintegration begins and the rate of advance of disintegration over the body vary within wide limits according to water-temperature, concentration of the reagent and alkalinity, but the course of the process is the same in all cases with the rare modifications described above.

Another species of *Stentor*, apparently *S. polymorphus*, gives the same results, but the course of disintegration is not so readily followed in this species because of the absence of pigment and the less conspicuous myonemes.

III. Hypotricha.

Several species of hypotrichous ciliates including two species of *Stylonychia*, *Euplotes* and two unidentified forms have thus far been tested by this method and with the same result in all cases, viz., death and disintegration begin at the anterior end and proceed down over the body to the posterior end. When the animals are placed in KCN they at once begin to swim backward and continue to do so until death and disintegration occur.

The process of disintegration is in all essential features similar to that in *Stentor* and consists in a dissolution of the structure and a swelling of the cytoplasm. The boundary between the disintegrated and intact regions is sharp and the disintegrative change proceeds uniformly down the body, the rate of advance varying in different individuals and with different temperatures and concentrations of reagent.

In *Stylonychia* the beginning of disintegration is marked by the melting down of the structures at the anterior end into a mass of uniform semi-fluid cytoplasm while the cilia elsewhere are still active and the animal is moving rapidly backward. The dissolution of structure extends backward over the body and the disintegrated mass often stretches out and is left behind in a long trail of granules and semifluid droplets as the animal moves backward: Movement continues in each part until the advancing wave of disintegration reaches it, when it suddenly breaks down and seems to dissolve into a formless mass. The posterior end continues to move until finally it too dissolves and movement ceases.

The advance of the disintegration wave and the sudden melting of each structure as the wave reaches it are striking phenomena and the uniformity of the whole process in different individuals and in different species indicates that it is associated with a fundamental feature of the constitution of these cells.

If the concentration of the cyanide is so low that the animals live for an hour or more before disintegration begins *Stylonychia* usually loses its flattened elongated form and becomes almost spherical, but movement still continues and the anterior end is still distinguishable by its structure. In such cases the disintegration, when it finally occurs, is more rapid and extends from anterior to posterior end in a few seconds.

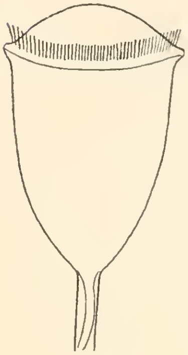
IV. PERITRICHA.

The forms examined include three species of Vorticellidæ and one of *Carchesium*. The results are practically identical in all cases. All of these forms are more resistant than *Stentor* and require a higher concentration of reagent. In *Carchesium* for example the course of death and disintegration in KCN

0.01 *m*. is as follows: The first change is a bulging of the peristome (Fig. 7) as in *Stentor*, but in these forms this does not usually occur until after the cilia have ceased or almost ceased to beat. This bulging is followed within a few seconds by disintegration of the peristome region including the cilia (Fig. 8), while the rest of the body is still intact and then a pause of a few seconds occurs. Disintegration of the body below the peristome is much more rapid than in *Stentor* and during the next few seconds the structure, except for the meganucleus, disappears completely and the entoplasm swells and spreads out into the water, the process beginning at the oral region and rapidly advancing aborally. But the ectoplasm does not disintegrate completely: a part of the body wall is elastic and as the contents of the body flow out this structure contracts and remains visible for a long time as a transparent skeleton or "shadow" of the body, much reduced in size but retaining more or less the original shape (Figs. 9 and 10). So far as can be determined the ectoplasm of the peristome region does not take part in the contraction but after the early stages of disintegration disappears completely. Evidently the lateral body wall is differentiated in part into a supporting substance which is no longer metabolically active. In this connection the difference in contractility between the peristome region and the lateral walls of the body in the living animal is of interest. The peristome is highly contractile and can be closed and opened while other parts of the body wall are capable of but little contraction. The contractile region disintegrates completely at once in KCN while the non-contractile region does not.

The stalk resembles the lateral body wall in its resistance but the contractile fiber usually shows disintegrative changes at about the same time as the peristome region or a little later. These changes consist in a loss of the regular contour, the appearance of varicosities and an apparent breaking up into a series of granules or droplets. Examination of the fiber with very high powers has not been made.

When lower concentrations, *e. g.*, KCN 0.002 *m*. are used, the death changes are limited to the appearance of hyaline or slightly granular droplets on the peristome region (Fig. 11) or to a bulging of the whole peristome (Fig. 12) and the body may remain otherwise intact for several days. The changes in ethyl ether 2 per cent. are very similar. In a small vorticellid of which only a few individuals were present in my cultures the death changes consisted of the bulging of the peristome (Fig. 13) and later of the disappearance of the cilia and the swelling of the cytoplasm until it formed a large rounded mass still enclosed within the stretched membrane of the peristome region (Fig. 14) while the lateral body-wall retained its form. In higher concentrations the changes in this form would undoubtedly be the same as in *Carchesium*.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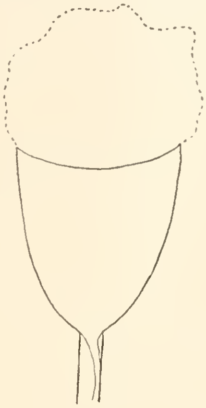
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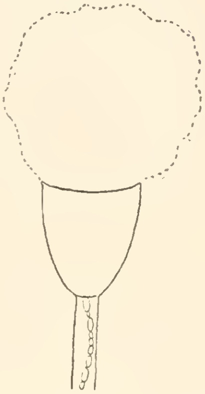
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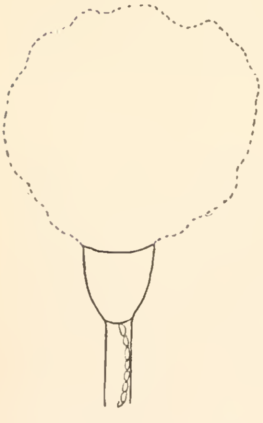
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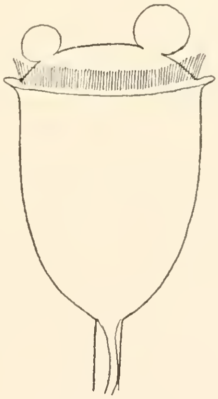
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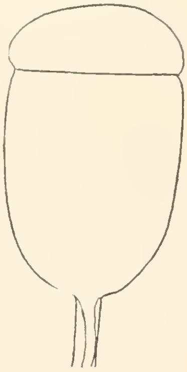
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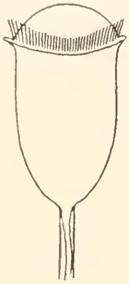
In all four species examined the peristome region is very evidently more susceptible to the reagent than other parts. In the remainder of the body the death changes do progress from the oral to the aboral end, but their progress is much more rapid than in *Stentor*. In KCN 0.01 *m.* disintegration is completed within 5–10 seconds after the pause following peristome disintegration. In these forms then the axial gradient consists first in a marked decrease in susceptibility from the peristome region to more aboral regions and a slight decrease in the rest of the body from the oral to the aboral end.



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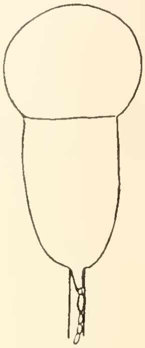

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V. Holotricha.

Paramœcium is not very susceptible to KCN, but the individual differences in resistance are considerable. In KCN 0.01 *m.* with water-temperature 22°–25° C. average survival time of animals from one culture was six or seven hours, but some animals die after two or three hours and some live eight or ten hours. Moreover, different cultures differ very considerably in resistance to cyanide.

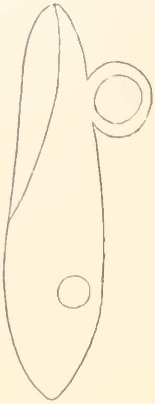
When the animals are placed in KCN they at once begin to swim backward. This continues for a period varying from a few minutes to half an hour or in some cases longer. Gradually the backward movement becomes less and less marked until the animal is simply revolving and then sooner or later it begins to swim forward once more and continues until it dies.

In KCN 0.01 *m.* the disintegration changes begin in the great majority of cases with the appearance of a rounded swelling over the anterior vacuole. The ectoplasm involved loses its cilia and its characteristic structure but a thin membrane remains surrounding the swelling. The swelling gradually increases in size and becomes a spherical mass into which the anterior vacuole often passes (Fig. 15). Here the vacuole undergoes enlargement and does not contract rhythmically but finally disappears. The vacuole is surrounded by granular cytoplasm. In other cases the anterior vacuole remains in its normal position and the swelling contains only granular cytoplasm (Fig. 16). In all cases the swelling is lighter in color than other parts of the body and the characteristic structure and the cilia of the region have disappeared.

Occasionally the swelling appears over the posterior instead of the anterior vacuole (Fig. 17) but this is comparatively a rare occurrence. In a few individuals among the thousands observed swellings of about equal size appeared over both vacuoles (Fig. 18) and in a few other cases a larger swelling over the anterior, a smaller over the posterior vacuole. But in at least nine out of ten the swelling appeared only on the anterior vacuole.

The swelling gradually enlarges, while the animal continues to swim, the vacuole disappears sooner or later (Fig. 19) and the intact portion of the body gradually decreases in size, the ectoplasm evidently undergoing contraction as the entoplasm gradually passes into the swelling (Fig. 20). At this stage the animal is still swimming, the structure of the ectoplasmic regions not involved in the swelling is intact and meganucleus and posterior vacuole retain their positions. As noted above the cytoplasm in the swelling, which is now as large as the remainder of the body, is lighter in color and more transparent and more uniform in appearance than in the intact regions. It is evidently dead and some of its constituents have probably diffused through the membrane as does the blue pigment in the case of *Stentor*.

The disintegrative change gradually spreads over the ectoplasm, less rapidly on the side of the oral groove than on the opposite side, and the intact ectoplasm continues to decrease in size (Fig. 21). The cilia on the intact portion are still present and beating at this stage and the animal may move slowly in a circular course with



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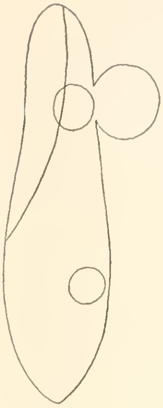

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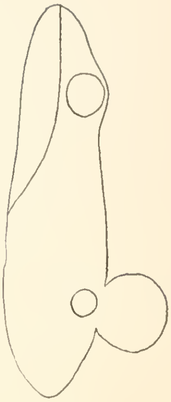

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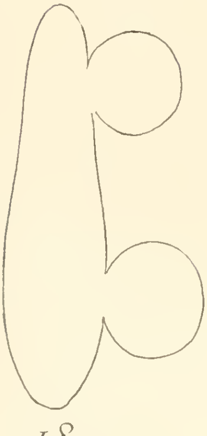

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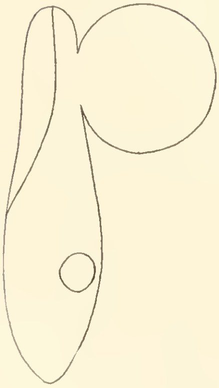

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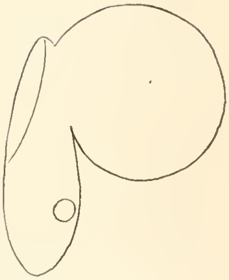

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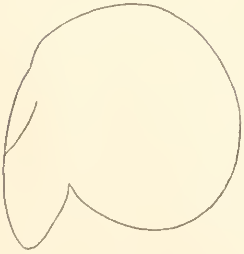

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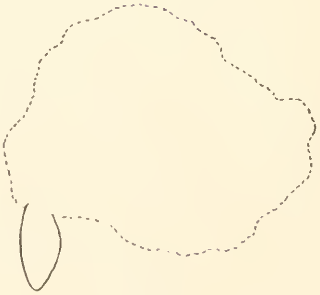

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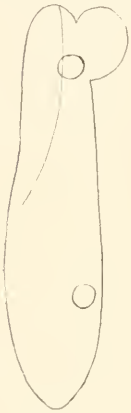
the swelling always toward the center. This direction of movement results from the distribution of the cilia and the weight of the swelling. In many cases the swelling bursts sooner or later, the contained cytoplasm flows out, a wave of disintegration accompanied by an increase in translucency passes rapidly over the remaining portions of the body, toward the posterior end, blotting out the ectoplasmic structure and the cilia and all that remains is the mass of granules spreading in the water, the meganucleus and a small transparent ectoplasmic "shadow" of the portions not directly involved in the swelling (Fig. 22).

In other cases the dissolution of structure occurs throughout the body before the rupture of the membrane and the body may retain the shape of Fig. 21 for some minutes but finally the membrane disappears and only a shapeless granular mass containing the meganucleus remains.

In higher concentrations of KCN or in lower concentrations with increased alkalinity disintegration is more rapid but its course is essentially the same. In KCN 0.02 *m.* or in KCN 0.001 *m.* which is made more alkaline by the addition of NaOH death and disintegration occur within ten to fifteen minutes. The course of disintegration is much the same as that described above, a small swelling containing granular cytoplasm lighter in color than the rest appears over the anterior vacuole, but the vacuole does not enter it in most cases (Fig. 23); this swelling increases rapidly in size and soon involves the anterior end of the body (Fig. 24); sooner or later its membrane disappears, the wave of disintegration spreads over the body, blotting out its structure and only a mass of granular protoplasm and the contracted "shadow" remaining (Fig. 25). In a few individuals droplets of semi-fluid hyaline substance appeared on other parts of the body surface and increased in size until bursting occurred.

In these solutions a swelling over the posterior vacuole appears much more frequently than in KCN 0.001 *m.* without the addition of alkali or even in 0.01 *m.* In most cases where a posterior swelling appears it is smaller than the anterior, but occasionally it is larger and rarely only the posterior swelling appears. In general, the higher the concentration of the reagent, whether with or without the addition of alkali, the more frequently the posterior as well as the anterior vacuole region swells. Increase in alkalinity accomplishes the same result as increase in concentration of KCN, *i. e.*, it increases susceptibility. In other words, when the concentration is so low that the animals die very slowly only the anterior vacuole region undergoes swelling before the death and disintegration of the rest of the body.

In still higher concentrations, *e. g.*, KCN 0.04 *m.* the animals die very soon, hyaline droplets appear on all parts of the body and in a few seconds nothing remains but the entoplasmic granules and the meganucleus more or less surrounded by the semi-fluid hyaline substance in rounded masses or droplets, apparently with a surface membrane. Sooner or later the droplets burst, apparently in consequence of rupture of the membrane, and the substance of the body spreads through the water. In such cases a gradient does not usually appear because the concentration is sufficiently high to kill all parts at the same time or nearly so.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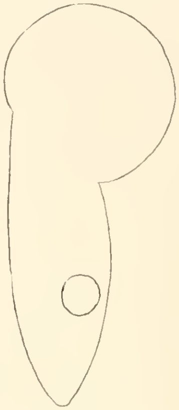
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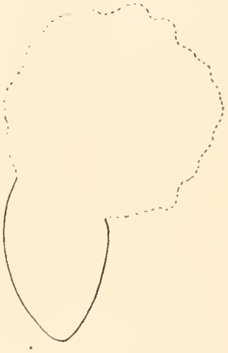
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Discussion.

It is evident from the preceding sections that a more or less definite axial gradient in susceptibility to KCN exists in all of the forms examined. These forms represent the four orders of ciliate infusoria, Heterotricha (*Stentor*), Hypotricha (*Stylonychia*, *Euplotes*, etc.), Peritricha (*Vorticella* and *Carchesium*) and Holotricha (*Paramæcium*), so that the existence of an axial gradient is evidently characteristic of the group.

If the susceptibility is in any degree a measure of the rate of metabolism or more specifically of oxidation processes as it unquestionably is in many other cases (Child 13*b*) we must conclude that an axial gradient in the rate of these processes is a characteristic feature of the ciliates. Moreover, the structure and behavior of the animals also suggest differences in rate of metabolic processes along the axis. In *Stentor* the peristome region is undoubtedly the most sensitive, most highly contractile and most active region of the body. In *Vorticella* and *Carchesium* a similar difference exists between peristome and body exclusive of the contractile fiber of the stalk. In *Stylonychia* the development and activity of cilia are greater in the anterior region than elsewhere. In the case of *Paramœcium*, however, the structure and behavior do not afford any definite evidence upon this point.

In forms like *Stentor*, *Vorticella* and *Carchesium*, where a special contractile peristome region is present, this region is distinguished from other regions of the body by a considerably greater degree of susceptibility, while in *Stylonychia* the gradient of susceptibility is almost uniform. Moreover in the elongated body of *Stentor* the gradient is steeper in the anterior than in the posterior half, *i. e.*, in the region which forms the stalk the gradient is relatively slight as compared with the body proper. In short there can, I think, be no doubt that the differences in susceptibility along the axis in *Stentor*, *Stylonychia*, *Euplotes*, *Vorticella* and *Carchesium* are an expression of a dynamic axial gradient, a gradient in the rate of certain fundamental metabolic processes.

The case of *Paramœcium* requires some further consideration. The constant association of the swelling with the vacuoles, and in the great majority of cases with the anterior vacuole alone raises the question whether we are to regard this region as possessing a higher rate of metabolism or of respiration than other parts of the body or whether some complicating factor, *e. g.*, weakness of the ectoplasmic wall in this region, plays a part in the localization of the swelling. There is no visible indication that the ectoplasm of the vacuolar region is thinner or structurally different in any way from that of other regions of the body, but such negative evidence is of course not conclusive. On the other hand the fact that the vacuole remains in its normal position at least as often as it enters the swelling indicates that the swelling is not simply the result of the enlargement of the vacuole and consequently stretching of the body wall. It should also be noted that when the swelling appears the ectoplasm does not merely stretch, it loses its structural features, including the cilia and the entoplasm which the swelling contains differs in appearance from that in other parts of the body. And finally, the vacuolar mechanism is very sensitive to cyanide, the pulsation ceases soon after the animal is placed in the solution and the vacuole slowly undergoes enlargement and sooner or later collapses and disappears. The vacuole region is apparently a specialized contractile mechanism and is undoubtedly the seat of a relatively high rate of dynamic activity. In short the only logical conclusion is that the greater susceptibility of the region where the swelling appears results from greater dynamic activity.

But a difference of some sort must exist between anterior and posterior vacuolar regions, for it was pointed out above (p. 47) that in the lower concentrations of the reagent only the anterior vacuolar region shows a swelling and that the frequency of swelling in the posterior as well as in the anterior vacuolar region increases as the concentration increases. Evidently the posterior vacuolar region is for some reason less susceptible than the anterior and a higher concentration is necessary at least in the great majority of individuals, to bring about swelling in it than in the anterior region.

A comparison of the rate of pulsation in anterior and posterior vacuoles affords a clue to the nature of this difference. I have found that in general the rate of the anterior vacuole is considerably higher than that of the posterior. Sometimes the ratio is as high as 5 : 3, in other cases less, but the difference in rate exists, at least in the great majority of individuals. Evidently the posterior vacuole has a lower rate of dynamic activity than the anterior, and the difference in susceptibility of the two vacuolar regions is undoubtedly associated with this difference in rate. In the lower concentrations or in individuals with a low susceptibility the special susceptibility appears as a rule only in the anterior vacuolar region because only here is the rate of reaction sufficiently high to bring about the rapid change. In higher concentrations the special susceptibility may appear in both vacuolar regions, but even in such cases the swelling usually appears earlier or is of larger size in the anterior region. In short the facts indicate that the two vacuolar regions of *Paramœcium* are more or less definitely localized regions of relatively high rate of metabolism or more specifically of respiration and, furthermore, that the rate in the anterior vacuolar region is higher than that in the posterior.

The lower susceptibility of the posterior vacuolar region to KCN and the lower rate of pulsation of the posterior vacuole in normal living animals suggest the existence of an axial gradient in rate of metabolism, and in the death and disintegration of the body in general this gradient appears as has been shown. When the disintegrative change begins to spread from the vacuolar region the anterior end of the body is attacked first and from this end the process of disintegration spreads downward to the posterior end. The differences in susceptibility along the axis are not as great as the differences between peristome and other regions in *Stentor* and *Vorticella*, in other words the axial gradient in rate is less steep in *Paramœcium* than in those forms.

A general relation seems to exist between the character of the gradient and the degree of morphological and physiological "cephalization" in these different forms. In *Paramœcium* the gradient is comparatively slight and apparently uniform except for the vacuolar regions. In *Stylonychia* and *Euplotes* the gradient is steeper, *i. e.*, the differences are greater but here also it is uniform or nearly so. In *Stentor* a distinct gradient, steeper in the more anterior regions than that in *Paramœcium*, extends throughout the length of the body, but the peristome region is distinctly marked off from other parts by its higher rate, *i. e.*, the gradient from the peristome region to other parts is steeper than the gradient in other regions. And finally in *Vorticella* and *Carchesium* the gradient between peristome and other regions is steep as in *Stentor* but in other parts of the body the gradient is slight. When we compare these facts with the differences in morphology and behavior of these different species and of the different regions of the body in each the general parallelism between the different groups of facts is strikingly evident. The degree of morphological and physiological "cephalization" in these infusoria parallels the degree of difference between the rate of metabolism in the apical region and that in other regions. The obvious inference from the facts is that the axial metabolic gradient has some very intimate relation to the morphological and physiological polarity of these organisms.

The occasional departures from the general rule such as the case observed in *Stentor* where disintegration in the posterior half of the body proceeded from the posterior end and the rare cases in *Paramœcium* where a swelling appears over the posterior vacuole only undoubtedly result from temporary stimulation of the posterior regions. The dynamic gradient is not to be conceived as fixed and invariable; it may be temporarily or in many cases even permanently eliminated. But wherever it is present to a certain degree and more or less continuously existent it must determine both morphological and physiological differences along the axis.

The facts of embryology indicate that a gradient in rate of metabolism exists or arises early in the embryonic development of every animal and that the region of highest rate becomes the apical or cephalic region. In plants likewise the apical region of the axis is undoubtedly the region of highest metabolic rate.

Attention has already been called in earlier papers (Child, '13*a*, '13*c*) to these and to other facts and further data will be presented in the future. The facts indicate as I believe that a gradient in the rate of metabolism or of certain metabolic processes is the dynamic basis of morphological and physiological polarity in organisms. The existence of such a gradient in unicellular organisms and the striking relation between its features and the morphological and physiological features of the different species in these as well as in multicellular forms is to say the least suggestive. It points very clearly to a fundamental similarity between unicellular and multicellular organization and differentiation.

These observations are also of some interest in another connection. Loeb ('99) has advanced the hypothesis that the nucleus is the chief organ of oxidation in the cell and has interpreted the inability of non-nucleated pieces or cells to regenerate to asphyxiation in the absence of the nucleus. Verworn ('91, '04), on the other hand, has shown that non-nucleated pieces of various ciliates are as susceptible to lack of oxygen and recover as readily when it is present as nucleated cells or pieces. These facts indicate that respiration takes place in the cytoplasm, even when the nucleus is absent. Later investigations by various authors support and confirm Verworn's view.

My own observations show that the nucleus is much less susceptible to cyanide than the cytoplasm. If the cyanides inhibit in some way the oxidation processes as most authorities believe the much greater susceptibility of the cytoplasm indicates that the oxidations are localized in it, at least to a much greater extent than in the nucleus. Moreover, the existence of a gradient in susceptibility to cyanide in the cytoplasm and particularly in the ectoplasm indicates that different rates of oxidation are localized in different regions of the unicellular body in very much the same way as in multicellular forms. But even if the cyanides produce their effect in some other way than by inhibiting the oxidation processes specifically, whether their action is upon some other reactions of the metabolic complex or upon the metabolic substratum of the cell, they make it evident that a dynamic gradient exists along the main axis of the unicellular ciliate infusorian body.

Summary.

1. An axial gradient in susceptibility to KCN is present in *Stentor*, *Stylonychia*, *Vorticella*, *Carchesium* and *Paramœcium.* This suceptibility gradient indicates the existence of a gradient in rate of metabolism or of oxidation processes.

2. In all forms examined the anterior or apical region is the highest point in the general gradient, although localized regions of still higher rate such as the vacuolar regions of *Paramœcium* may exist.

3. A close parallelism exists between the character of the gradient and the morphological and physiological features of the species. Where the gradient is uniform and slight, structural and physiological differences along the axis are slight and where

the changes in steepness are great, marked morphological and physiological differences are present.

4. The facts indicate that the axial gradient is the dynamic basis of morphological and physiological polarity in the ciliate infusoria as well as in other organisms.

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Vol. XXVI. *February, 1914.* *No. 2*

BIOLOGICAL BULLETIN

STUDIES ON THE PHYSIOLOGY OF REPRODUCTION IN THE DOMESTIC FOWL.

VI. Double- and Triple-Yolked Eggs.[1]

MAYNIE R. CURTIS.

The most common type of abnormal egg produced by the domestic fowl is the double-yolked egg. Eggs with three yolks, however, are very rare. The purpose of the present paper is, first, to record some observations on the frequency of the occurrence of double- and triple-yolked eggs and the relation of their production to the age of the bird; and, second, to consider so far as possible the nature of the processes involved in their formation and the contribution that such a study makes to our knowledge of the physiology of egg production.

The Frequency of Occurrence of Double- and Triple-Yolked Eggs and the Relation of Their Production to the Age of the Bird.

During the last six years only three triple-yolked eggs are known to have been produced by the flock of birds belonging to the Maine Agricultural Experiment Station. The poultrymen have been under instructions to bring all abnormal eggs to the laboratory where they are opened. It seems hardly probable that they would fail to observe the abnormal size of a triple-yolked egg. During the six years more than three thousand different adult birds have been kept, each for one or more years. If no triple-yolked eggs have escaped notice, fewer that one bird in a thousand have laid triple-yolked eggs.

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[1] Papers from the Biological Laboratory of the Maine Agricultural Experiment Station, No. 56.

The previous papers in the series of "Studies on the Physiology of Reproduction in the Domestic Fowl" are:

I. Pearl, R., "Regulation in the Morphogenetic Activity of the Oviduct," *Jour. Exp. Zool.*, Vol. 6, pp. 339–359, 1909.

II. Pearl, R., and Surface, F. M., "Data on the Inheritance of Fecundity Obtained from the Records of Egg Production of the Daughters of 200-egg Hens," Me. Agr. Exp. Sta. Ann. Rept., 1909, pp. 49–84.

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IV. Pearl, R., and Surface, F. M., "Data on Certain Factors Influencing the Fertility and Hatching of Eggs," Me. Agr. Exp. Sta. Ann. Rept., 1909, pp. 105–164.

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Each of the three triple-yolked eggs produced in these flocks was laid by a different bird. In each case the triple-yolked egg was one of the first six eggs produced by a pullet which began to lay when between five and six months old. This indicates that young pullets are more likely to produce this type of abnormal egg than are older birds. Fowls show also a greater tendency to lay double-yolked eggs at this age than when more mature. At this stage of development the time of successive ovulations is less precisely regulated than later in life. It should be kept in mind, however, that even at this age there are many more birds which do not lay multiple-yolked eggs than there are which do, and that usually one bird lays only one such egg.

Data collected during two investigations in the physiology of egg production show incidentally what percentage of the pullets which began to lay when between five and seven months of age produce eggs with more than one yolk. The first of these observations was made in the fall of 1910. At that time data were taken on all the eggs laid by a small flock of pullets. Twenty of these pullets began to lay when under seven months of age. Among the first ten eggs of four (or 20 per cent.) of these there was one or more which was double-yolked. The second observation was made in the fall of 1913 when one hundred and sixty-nine pullets laid before November 1. All of these were less than seven months old. Thirty-three of them or 19.5 per cent. laid one or more eggs with more than one yolk. The data on the laying of multiple-yolked eggs by these two flocks of pullets are brought together in Table I.

This table shows that in 80 per cent. of the birds of this age the normal rhythm of ovulation, and the successive stages of egg formation which result in the enclosing of each yolk in

TABLE I.

SHOWING THE NUMBER OF MULTIPLE-YOLKED EGGS PRODUCED BY PULLETS FIVE TO SEVEN MONTHS OLD.

No. of Eggs with More than one Yolk.	No. of Pullets Observed.	Percentage of Observed Flock.
0	152	80.42
1	26	13.76
2	8	4.23
3	3	1.59

separate envelopes, are sufficiently well regulated at the beginning of laying to result in the production of only normal single-yolked eggs. The other 20 per cent. of the birds produce one to three eggs with more than one yolk. Less than 6 per cent. of the flock, however, show more than one such irregularity while only about 1.5 per cent. show three such imperfections of regulation. It is thus clear that even in young birds ovulation and egg formation is in general a well-regulated process. Disturbances in the rhythm are, however, most likely to occur among young birds.

The question naturally arises as to whether it is the immaturity of the bird or the lack of laying experience which is responsible for the unstable regulation of ovulation. If birds which begin to lay younger show a more decided tendency to produce eggs with more than one yolk while those which are more mature produce only normal single-yolked eggs, it might be concluded that immaturity is associated with a lack of precision in the regulation of the time of successive ovulations.

Data now being collected at this laboratory will probably be sufficient to answer this question. The evidence at present available, although insufficient to settle the question, suggests that immature birds produce more multiple-yolked eggs than mature birds which have not laid when young. The evidence consists of the three following observations. First, only about one-third of the pullets of all sorts, including many crosses not carrying hereditary factors for high winter production, which are kept at the station, begin to lay before November 1. The others begin to lay at varying times up to March. A few double-yolked eggs are produced in every month of the year, but by far the greatest number are produced at the end of September and during October when the pullets which are coming on to lay are immature. This observation does not agree with the statements of Immerman,[1] Panum[2] and Parker[3] that the greatest number of double-yolked eggs occur in the warmer part of the year. Immerman, however, recognized the fact that the seasons when he obtained the largest number of double-yolked eggs coincided with the periods of highest egg production. He believed that the laying of double-yolked eggs occurred during the entire laying period and that no season had an especial influence on their production. Since the eggs on which these workers base their statements were collected from the market, or brought to them by their friends, it is likely that they did not receive the double-yolked pullet eggs, which are often not distinguishable externally from the single-yolked eggs of the year-old and two-year-old hens.

It is also possible that the birds in the part of the country where these people worked were so bred and handled that they did not begin to lay until much later than birds specially bred for winter production.

The second observation which suggests that an immature bird is more likely to produce double-yolked eggs than one which is mature was made upon the eggs of a single pullet which began to lay when she was exactly three months old and before she had assumed her adult plumage. The first ten eggs of this pullet were kindly presented to this station by the owner of the bird, Mr. Walter Gerald, of Unity, Maine. All of these ten eggs were smaller than a normal pullet's egg but the four largest of them contained two yolks. This pullet, which began to lay when much less mature than any of the pullets which have been raised on the station plant, produced more double-yolked eggs than have been laid by any of these birds. This bird may, of course, be absolutely abnormal in respect to the production of double-yolked eggs.

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[1] Immerman, F., "Über Doppeleier beim Huhn," Inaugural-Dissertation, Basel, 43 pp., 1899.

[2] Panum, P. L., "Untersuchungen über die Entstehung der Missbildungen zunächst in den Eiern der Vögel," Berlin, 260 pp., 1860.

[3] Parker, G. H., "Double Hen's Eggs," *American Naturalist*, Vol. XL., pp. 13–25, 1906.

It might also be repeated here that the three triple-yolked eggs were all produced by pullets less than six months old.

The third observation is that only normal single-yolked eggs were produced by three pullets which did not lay until they were 8¾ to 9 months of age. Every egg of these birds was opened. Since 80 per cent. of the pullets between 5 and 7 months of age lay only normal single-yolked eggs, this third observation has no statistical value, but it is given since it is the only absolutely accurate data at present available on the eggs of the birds which begin to lay when fully mature.

The kind of disturbances which result in the formation of double- and triple-yolked eggs are not yet thoroughly understood. From the preceding paragraphs it would appear that whatever their nature they are more apt to occur in immature than in mature birds. Yet during six years observation, the Experiment Station's flock, consisting each year of from 600 to 800 birds, has produced double-yolked eggs during every month of the year. This accords with Immerman's observations in Basel and indicates that such disturbances may occur at any time.

Parker quotes Landois'[1] statement that the laying of double-yolked eggs may become habitual with certain hens and also cites the single cases of such birds noted by Bartels[2] and Immerman.[3] A third single case has since been reported by Glaser.[4] Not a single case of this kind has occurred among the large number of birds owned by this station during the last six years. This fact together with the small number of the actual cases cited in the literature indicates that birds which habitually lay double-yolked eggs are very rare. The occasional occurrence of such cases, however, indicates that physiological disturbances capable of causing such a result may become chronic.

Data from the station flock, however, indicate that even when the disturbances are not chronic they may recur several times in one individual, *i. e.*, an individual may show a predisposition to them. Table I. showed that some pullets before they were seven months old had produced as many as three eggs with more than one yolk, while 80 per cent. of the flock had laid only single-yolked eggs. Also the data collected for the investigation on egg size contribute some evidence on this point. All of the eggs laid by 22 birds during their first two laying years were opened. Only 5 of these birds ever laid an egg which had more than one yolk. Four of these five laid one or two double-yolked eggs among their first seven. Two of the four never afterward laid an abnormal egg. The others, however, produced two (in the first case) or three (in the second) double-yolked eggs when they were mature. Out of the flock of 22 only one bird which had not produced double-yolked eggs at the beginning of the first laying period produced one after she was mature. This indicates that individuals differ in the stability of the regulation of their reproductive processes, and if these processes in an individual are unstable, irregularities are most likely to occur among the first eggs produced when the bird begins to lay while young. Also different individuals show varying degrees of instability, so that some may also show irregularities later.

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[1] Landois, "Missbildungen bei Hünner-Eiern," *Zool. Garten*, Jahrg. 19, pp. 17–24.

[2] Bartels, M., "Hühner Ei mit Zwei Dottern," Sitzungs-Ber. Gesellsch. naturf Freunde Berlin, Jahrg. 1895, pp. 143–145.

[3] Immerman, *loc. cit.*

[4] Glaser, O., "The Origin of Double-Yolked Eggs," BIOL. BULL., Vol. XXIV., pp. 175–186.

The Relation of the Production of Double-Yolked Eggs to Other Forms of Abnormally Rapid Egg Production.

A study of the nature of the disturbances which cause the formation of double- and triple-yolked eggs is limited by the fact that as yet we do not fully understand the nature of the regulation of the egg-forming processes which cause the production of successive single-yolked eggs. One of the earliest and most extensive investigations of the physiological processes involved in egg formation was that of Coste.[1] He made important observations on the time spent by the egg in the oviduct. Similar observations are also recorded in a previous paper from this laboratory.[2] Published and unpublished observations show that normally a yolk is ovulated into the mouth of the oviduct and passes down the duct, receiving the chalazal layer, chalazæ, albumen, shell membrane and shell. The egg remains in the oviduct several hours after the shell is formed. In the case of a few birds which were laying every day a second yolk has been found in the upper part of the oviduct while there was still a hard shelled egg in the shell gland. These birds were killed after they had gone on the nest but before they had laid. That is, when birds are laying at daily intervals two eggs may be normally present in the duct at the same time. In order that two eggs may be separate single-yolked eggs it is only essential that the second does not overtake the first. If the two eggs keep moving toward the cloaca the time between successive ovulations may be somewhat reduced without causing any form of doubling of the egg. What happens is a reduction in the time between the laying of the eggs. Ordinarily the period between two eggs of a clutch is somewhat more than 24 hours. However, some of the best laying birds at the height of their reproductive period lay in less than 24-hour periods. For several days they may lay somewhat earlier each day than on the preceding day.

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[1] Coste, M., "Histoire du développement des corps organisés," Tome I., Paris, 1874.

[2] Pearl, R., and Curtis, M. R., "Studies in the Physiology of Reproduction in the Domestic Fowl, V., Data Regarding the Physiology of the Oviduct." Jour. Exp. Zool. Vol. 12, pp. 99–132.

One egg every day is the maximum normal egg production. Occasional cases have been reported where two eggs were produced at different times during the same day. Most such cases do not bear critical investigation. For example, at this and other poultry plants where trap-nests are used, a bird which is already credited with an egg on that day is sometimes found apparently to have laid again. The probability that the bird has actually laid two eggs is much smaller than the probability that the poultryman misread the first band number or some other slip occurred. Unless there is some independent check the evidence cannot be accepted. Such a check was possible in several such cases at this plant. One may be mentioned here. The eggs of bird No. 771D were being saved for incubation and each was marked with the bird number when it was taken from the trap-nest. Therefore, when the second egg was taken from the nest on May 19, 1908, the first egg had not been mixed with the eggs of other birds. The resemblance between the two eggs was sufficient to make it certain that the same bird had produced them. This bird had laid on each of the three preceding days, but did not lay on either of the two following days. The production of the two eggs in one day in this case may have been due to a shortening of the time between the last two ovulations in the clutch although it is also possible that it was due to the early expulsion of the last egg.

An authentic record of a White Wyandotte pullet which showed a rate of production high enough to cause several instances of the production of two eggs in a day has been published by Drew.[1]

Other cases have been recorded where two separate eggs have been produced at the same time. As in the preceding case all such records must be most critically investigated since an egg may have been overlooked the last time the nest was used or a bird may have entered a nest, laid and gone out while another bird was standing on the open trap door. A second bird may later be found in the nest with two eggs. Therefore, no record of two eggs at one time is accepted at this plant unless the two eggs so closely resemble each other and differ so decidedly from the average eggs of the breed that there can be no doubt that they were produced by the same bird. Four such undoubted cases of the laying of two separate normal eggs at one time have occurred at this plant. Two of the birds producing these had laid on the preceding day but did not lay on the following. These cases then must be accounted for by the unusually rapid succession of the second egg. One of the birds produced an egg on both the preceding and following days. In this case also the only possible explanation is an abnormally rapid period of egg production. In the fourth case the bird produced an egg on the following but not on the preceding day. This case may represent abnormally rapid production, or it may simply be that the first egg failed to be discharged from the duct until the second one was completely formed. These cases all show that an egg may overtake its predecessor after they are both completely formed. In some cases this is most probably due to a decrease in the normal minimum time between ovulations, although the same effect (*i. e.*, production of two eggs at the same time) is produced by the holding of the first egg in the duct after it would normally be laid.

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[1] Drew, Gilman A., "Hens that have Laid Two Eggs in a Day," *Science*, Vol. 26, N.S., pp. 119–120, 1907.

A few cases of egg-bound birds have come to autopsy in which the uterus contained a second fully mature egg. These cases do not of course necessarily indicate an unusually rapid egg production. One case, however, came to autopsy where there were two normal thin shelled eggs in the uterus. Neither one seemed to have the shell completely formed. There was no evidence that the eggs could not be laid. This appeared to be a case where two eggs were being completed at the same time. The bird had never laid.

These cases have been described because it seems clear that the kind of disturbances which cause the production of eggs with more than one yolk may also, when slighter in extent or when localized in a more posterior portion of the oviduct, cause other irregularities in egg production.

There is in fact a certain type of abnormal egg which forms the logical step between two eggs at one time and the double-yolked egg. Such eggs, although occurring very infrequently, have several times been described in poultry journals. One of them has been produced at this plant. These abnormalities consist of pairs of normal eggs united by a tube of membrane containing albumen. In the pair of such eggs produced at this plant the components were both soft shelled, one of them having a little more shell than the other. In such cases the second egg must have so nearly approached the first that the stimuli upon the isthmus glands overlapped, causing the formation of the membranous tube continuous with the shell membrane of each. It is interesting to note that the bird which produced this egg had produced a double-yolked egg six days before. Mr. F. E. Field,[1] of Birmingham, England, who described and published a photograph of such an egg, also notes that the pullet which produced it "has since laid several double-yolked eggs." The relation between such eggs and certain types of double-yolked eggs is obvious. If the second egg actually overtakes the first before the first has completely entered the isthmus a double-yolked egg will result. The relation of the two yolks to each other and to the surrounding egg envelopes will depend upon the particular part in the duct above the isthmus ring where the two eggs join, and upon the nature of the processes which bring the two eggs together.

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[1] Field, F. E., "Peculiar Twin Eggs," *Poultry World* (London), Vol. II., N.S., pp. 1152, 1913.

The Nature of the Disturbances in Normal Egg Production which Bring two Yolks together in the Oviduct, and the Influence of the Part of the Duct where they Join upon the Relation of the Yolks to Their Enclosing Envelopes.

Parker,[1] in his clear and logical discussion of double hens eggs, explains the cause of double-yolked eggs as the simultaneous discharge of two yolks either from the same or separate ovarian follicles. This is the most simple explanation of the class of double-yolked eggs where the two yolks have apparently passed the entire length of the duct together, as evidenced by the enclosure of the two yolks in a single set of envelopes, beginning with the chalaziferous layer. In such cases the two yolks are often very much flattened together and held by a thin, firm chalaziferous membrane. These yolks have a single set of chalazæ and are enclosed in a single envelope of thick albumen. A large number of eggs of this type are produced by the station flock every year. In this group would fall eggs belonging to both of the sub-classes of double-yolked eggs described by Immerman,[2] that is, those in which the yolks are enclosed in a single vitelline membrane, and also those in which the yolks have separate vitelline membranes. In fact all of the eggs of the first class must fall in this group. The occurrence of two yolks within the same vitelline membrane is, however, very rare among the eggs laid by the station flock. There has indeed been but one such case. In the other apparent cases it was possible by very careful dissection to remove the chalaziferous layer or chalazal membrane and separate the two yolks.

While Parker's explanation is sufficient to account for this group of double-yolked eggs it does not account for double-yolked eggs where the two yolks have some separate envelopes. Nor indeed is it the only possible explanation for double-yolked eggs where all the envelopes are common to the two yolks.

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[1] *Loc. cit.*

[2] *Loc. cit.*

Glaser[1] suggests that a failure of the peristalsis of the duct just below the infundibulum, due either to "a deficiency of substance normally inducing the movements or (to) the subnormal irritability on the part of the oviduct," might result in a yolk remaining in the upper part of the duct until a second was normally ovulated. The two might then pass down the duct together. Whether or not this ever happens is not known, but it is, certainly, theoretically possible.

Glaser's second suggestion as to an additional cause for the formation of double-yolked eggs is based upon the case he describes where the laying of double-yolked eggs was "habitual." The suggestion is that in such cases the laying of double-yolked eggs may have a "distinct ovarian basis" which in this case he believes to be the tendency to a secondary fusion of follicles which results in a common blood supply. This seems rather to be a suggestion of the nature of the underlying cause of the synchronous maturity and discharge of two or more yolks. The fusion of follicles and a common blood supply may be an important cause for simultaneous ovulations.

There is, however, one additional possibility for the formation of double-yolked eggs with a single set of egg envelopes. A yolk may be ovulated into the body cavity and remain in that portion of it which is walled off into a sort of ovarian pocket[2] formed by the mesentery, cœca, and air sac wall. It is thus near the mouth of the oviduct. If this free yolk entered the infundibulum just before or immediately after a second ovulation the two yolks might pass down the duct together. A free yolk has never been seen entering the oviduct but many observations of ovulation into the body cavity and the anatomical relation of the infundibulum to the ovary make it necessary to list this among the theoretically possible causes for the presence of two yolks in the duct at the same time.

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[1] *Loc. cit.*

[2] This pocket has been described by the author in a previous paper. Curtis, M. R., "The Ligaments of the Oviduct of the Domestic Fowl," Ann. Rept. Me. Agr. Exp. Sta., 1910, pp. 1–20.

The two yolks of a double-yolked egg often possess some separate envelopes, indicating that they have not passed through the entire length of the duct together. In fact a careful study of a large number of double-yolked eggs indicates that the second may join the first at any point between the infundibulum and the isthmus ring.

Two separate yolks with a common set of envelopes may be so closely united by the chalaziferous layer that they appear to have also a common vitelline membrane. Such pairs of yolks are very much flattened together. There is a complete and graded series from this condition to one where the two yolks barely touch one another. In the latter case the two yolks have really chalazal membranes which are practically separate although they still have but one set of chalazæ. The two yolks have evidently passed down the duct in quick succession but the first has preceded the second sufficiently to allow each to preserve its form.

This group of double-yolked eggs passes insensibly into the group in which the yolks have separate chalaziferous layers but all the other envelopes in common. Fig. 1 shows diagrammatically the typical relations of the two yolks in double-yolked eggs where the yolks have common albumen envelopes.

Where the arrangements of the two yolks and the chalazæ are those shown in Fig. 1, *c* to *f*, the second yolk must have overtaken the first after the chalazal layer was formed and before the thick albumen was secreted; that is, near the beginning of the albumen secreting portion of the duct. In the cases figured the stimulations given by the two yolks upon the glands which cause the formation of the chalazæ must have overlapped. In cases like Fig. 1, *f* the chalazæ sometimes appear thin in the middle. This may be due to the faintness of stimulation on the glands or to stretching of the chalazæ as the yolks are bent on their axes. A few double-yolked eggs with one set of albumen envelopes have been observed where the two yolks had separate chalazæ and several where one yolk had two and the other had none that were visible. It should be stated that in all types of double-yolked eggs the chalazæ of the second yolk, (*i. e.*, the one nearest air cell of the egg) are often less well developed than those

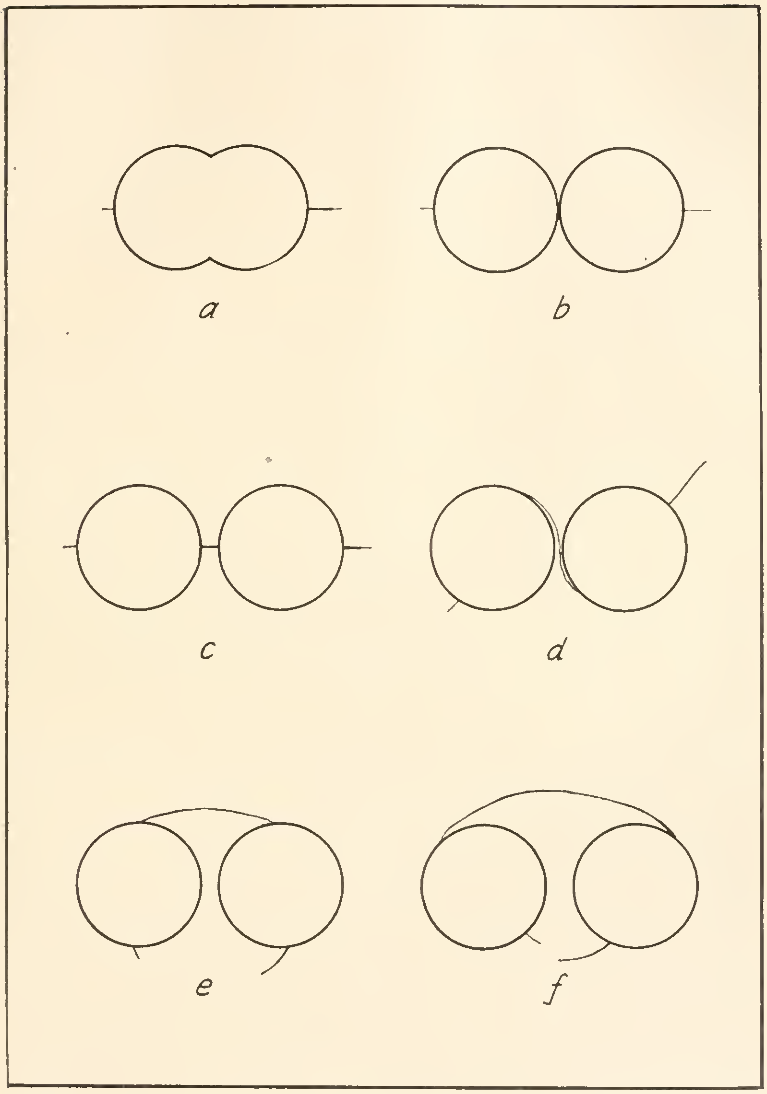

FIG. 1. Diagrams showing the various relations of the two yolks in double-yolked eggs where the yolks have common albumen envelopes. *a*, two yolks of a double-yolked egg which have all the envelopes in common; *b*, the form transitional between a double-yolked egg where the chalazal membrane is common to the two yolks and where each has a separate chalazal membrane; *c*, *d*, *e* and *f*, showing arrangements of the two yolks which have been observed in double-yolked eggs where the yolks have separate chalazal membranes but common albumen envelopes.

of the first. It is not always possible to determine the relation of the two yolks by this means. The many cases where this is possible, however, show that the chalazal axes of the two yolks are related to each other in various ways. Immerman[1] has shown by incubating double-yolked eggs that this is also true of the embryological axes. A study of double-yolked eggs suggests that this variation in position of the axes may in part result from the difference in the time elapsing between the entrance of the two yolks into the duct.

The next group of double-yolked eggs includes the transitional form between the eggs that have yolks with separate chalazæ and a single thick albumen envelope and those with yolks that have separate albumen envelopes. This group is not always easily distinguished from the last group and it is often impossible to decide whether or not one or both of the yolks have some separate layers of albumen. There are, however, cases where there is certainly considerable thick albumen between the two yolks although both are enclosed in a common mass of thick albumen. This type of egg will be produced whenever the second egg overtakes the first in the albumen secreting portion of the duct.

The last group of double-yolked eggs to be considered contains those which have separate thick albumen envelopes. This group is easily distinguished and is not uncommon. Seven of the 44 double-yolked eggs produced by the station flock during the month between September 20 and October 20, 1913, belong to this group. These eggs can usually be distinguished by the shape of the shell which has more or less of a depressed ring around the center. The double nature of the egg is visible externally. The depressed ring is sometimes hardly visible, or again it may be very pronounced. In cases where the depression is slight there is usually only a smooth membrane inside the shell, but in cases where it is more pronounced there is sometimes at the base of the depression a very narrow shelf of membrane which extends all or part of the way around the shell. There can be no doubt that in this group the two eggs join while passing into theisthmus and that the depth of the depression depends upon the time during this passage when the two eggs unite.

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[1] *Loc. cit.*

The posterior of the two eggs (that is, the one toward the air cell in the shell membrane) has a less deep but more viscous envelope of thick albumen than the anterior one. In consequence the anterior portion of the shell is larger. The relative position of the chalazal axes of the two yolks cannot always be determined, since, as noted above, the chalazæ of the second yolk are often not distinguishable. The chalazæ of the first yolk are always, so far as observed, in the same position as in a normal single-yolked egg. A few cases have been observed where the chalazal axis of the second egg was also in normal position. In one case, however, where the chalazæ of the second yolk were visible, the chalazal axis was turned about 30° from normal.

This group of eggs is the end of the series of double-yolked eggs, since when an egg overtakes its predecessor after that egg has passed entirely into the isthmus, the result is no longer two eggs within one membrane and shell. The relation of this group to twin eggs connected by a membranous tube is obvious.

In all double-yolked eggs where one or more of the egg envelopes is not common to the two yolks, it seems that instead of the simultaneous entrance of two yolks into the oviduct, the first precedes the second by a short time. This is probably most often due to the ovulation of a second yolk at a short interval after the first. It may sometimes be due to the fact that either the first or second yolk to enter the duct was ovulated into the body cavity and was taken up by the duct shortly before or immediately after the next ovulation. Or perhaps the first egg may sometimes come to a place in the oviduct which is subnormally sensitive to peristaltic stimuli, and remain there until joined by the second yolk. There is also a fourth possibility, namely that antiperistaltic movements may carry the first egg back up the duct until it meets the second.

Parker[1] called attention to the fact that the occurrence of antiperistalsis of the oviduct is proven in the cases where fully formed eggs are found in the body cavity. He further shows that only antiperistalsis can account for the enclosure of an egg within an egg. If an egg which has received its shell membrane, or its shell membrane and shell, is carried back up the duct and there meets another, and the two come down together, a double egg (*ovum in ovo*) will be formed. If, however, an egg which has not received its shell membrane is carried back up the duct and meets another coming down the result will be some form of double-yolked egg, the particular sort depending upon the relative development of the two eggs when they unite. The conditions under which an egg without a shell membrane would find another egg in the duct even if returned by antiperistalsis are not usual, since a second yolk does not normally enter the duct until some time after its predecessor has received a shell membrane. Such conditions might be brought about either by the entrance of a second yolk at an abnormally short interval after the first or by the first egg remaining stationary for some time or returning slowly.

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[1] *Loc. cit.*

It has been shown that the conditions necessary for the formation of a double-yolked egg (or a double egg) are that the two eggs unite in the oviduct and then proceed through the duct together. In all cases where the structure of the egg shows that the two components have not passed the entire length of the duct together the passage of the second egg must have been more rapid than that of the first for should the two eggs continue to move through the duct at the same rate they would only unite in the cases where their entrance was practically or absolutely simultaneous. In the preceding paragraphs the possibility of the delay or return of the first egg has been discussed. One further possibility is that the second yolk may pass through the duct at an abnormally rapid rate.

It is thus possible that double-yolked eggs do not always represent a simultaneous or abnormally rapid succession of two ovulations since a yolk may sometimes remain in the oviduct, or body cavity, until the time normal for a second ovulation. There are some cases of double-yolked egg production, however, which can only be explained by the occurrence of two or more ovulations at unusually short intervals. Such cases are seen when double-yolked eggs are produced on successive days, and when a double-yolked egg is laid after a long series of normal daily eggs. Several records of this kind have been obtained from the station flock.

TRIPLE-YOLKED EGGS.

The same processes which cause the formation of double-yolked eggs may also cause the formation of triple-yolked eggs. The number of triple-yolked eggs, however, is so small that an extensive study of them is impossible. The first of the triple-yolked eggs produced by the station flock was fully described by Dr. Raymond Pearl.[1] He called attention to the size relation between this egg and double-yolked, single-yolked and yolkless eggs of the same breed (Barred Plymouth Rock), and showed that the parabola

$$W = 16.242 + 43.762\,Y - 4.450\,Y^2$$

gave an extremely close fit to the observations.

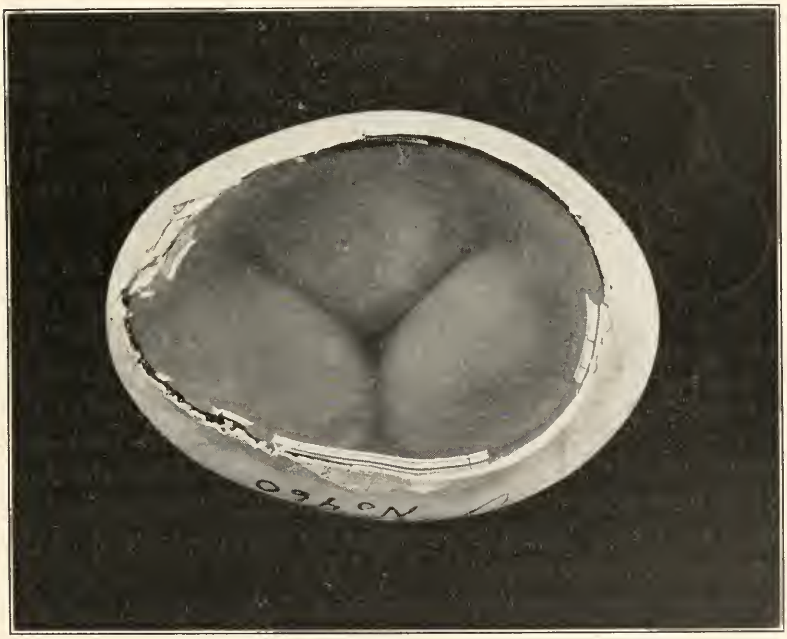

FIG. 2. Triple-yolked egg.

He describes the internal characters of the egg as follows: "Each yolk was enclosed in a separate yolk membrane. While the three yolks were in contact with each other, they were in no way fastened together. All of the yolks were of normal size, and of approximately the same size . . . the two kinds of albumen (of thick and thin consistency) which are normal were present in this egg. There was no trace of a chalaza in connection with any yolk or at either pole of the egg. The shell membranes were entirely normal."

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[1] Pearl, R., "A Triple-Yolked Egg," *Zool. Anz.*, Bd. XXXV., pp. 418–423.

The second triple-yolked egg was produced September 16, 1913, by bird No. 65K. The egg appeared externally like a large double-yolked egg. Its dimensions were as follows:

Length—63.6 mm.
Breadth—47.6 mm.
Weight—79.62 gms.

This was the first egg laid by the young pullet and was much smaller than the egg described by Pearl.

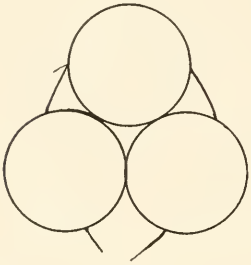

FIG. 3. Showing the arrangement of yolks in the triple-yolked egg.

A photograph of the internal arrangement of the egg parts is shown in Fig. 2.

The three yolks lay close together in a common thick albumen envelope. The chalazæ were feebly developed but their relations are shown in Fig. 3.

The yolks evidently entered the oviduct at successive short intervals and came down the funnel region in "Indian file". There was a short end of chalazal fibers at the caudal end of the second yolk but other fibers were continued to the third yolk as shown in the figure. Since the three yolks were close together in a common thick albumen envelope they must have joined at or very near the anterior end of the albumen secreting region.

Normal thin albumen and a normal shell membrane and shell were present.

The weights of the parts of this egg were as follows:

Weight of yolks	9.61 gms. 9.45 gms. 9.22 gms. 28.28 gms.
Weight of albumen	44.24 gms.
Weight of shell	7.10 gms.

Percentage of yolk	35.52
Percentage of albumen	55.56
Percentage of shell	8.92

The third triple-yolked egg was produced Oct. 8, 1913, by bird No. 16K. It was her sixth egg. The dimensions were as follows:

Length—65.2 mm.
Breadth—46.2 mm.
Weight—77.41 gms.

The egg was opened and the three yolks were seen lying in practically the same position as those in the triple-yolked egg of 65K. In an attempt to get a photograph of this egg the edge of the shell was accidently pressed against two of the yolks and yolk flowed out into the albumen. The yolk which was not broken and the shell were weighed. More data could not be accurately obtained but on the assumption that the yolk saved represented a mean of the three yolks the data for this egg are as follows. (The calculated data are in italics.)

Weight of yolk	= 10.29 × 3 = *30.87* gms.	Percentage of yolk......	*39.88*
Weight of albumen	= *30.17* gms.	Percentage of albumen..	*50.60*
Weight of shell	= 7.37 gms.	Percentage of shell......	9.52

The Size and Proportion of Parts in the Single-, Double- and Triple-Yolked Eggs of the Same Individual.

On September 28 and 29 the bird 65K which had laid the second triple-yolked egg laid normal single-yolked eggs. These eggs were not preserved. On October 2 she laid a double-yolked egg. Complete data were taken on this egg.

It was apparent that this bird's eggs offered an excellent opportunity to study the proportion of the parts in multiple eggs when compared with normal eggs produced by the same individual. Accordingly data were taken on the next ten normal eggs. On October 6 the bird produced another double-yolked egg. This was her seventh egg. Both of the double-yolked eggs of this bird had separate chalazal membranes but a common thick albumen envelope.

The data on the triple- and double-yolked eggs and the mean for the ten normal eggs are given in Table II.

The data given in this table show that the double-yolked eggs of this bird are not twice, nor the triple-yolked eggs three times, the size of the normal single-yolked eggs. This agrees with

TABLE II.

SHOWING THE DATA ON THE TRIPLE-YOLKED EGG, THE TWO DOUBLE-YOLKED EGGS AND THE MEAN OF TEN NORMAL EGGS OF 65K.

Egg No.	Date.	Length, Mm.	Breadth. Mm.	Index, Per Cent.	Weight of Egg, Gm.	Weight Albumen, Gm.	Per Cent. Albumen.	Weight Yolk, Gm.	Per Cent. Yolk.	Weight Shell, Etc., Gm.	Per Cent. Shell, Etc.
1	Sept. 26	63.6	47.6	74.84	79.62	44.24	55.56	9.61 9.45 9.22 28.28	35.52	7.10	8.92
4	Oct. 2	60.0	44.4	74.00	66.48	37.28	56.08	11.10 11.60 22.70	34.15	6.50	9.78
7	Oct. 6	59.7	45.5	76.21	69.67	39.05	56.05	11.80 11.65 23.45	33.66	7.17	10.29
Mean of double-yolked eggs.		59.85	44.95	75.11	68.08	38.17	56.07	23.08	33.91	6.84	10.04
Mean of 10 normal eggs.	Oct. 3 to Oct. 17	52.29	40.52	77.53	49.11	31.23	63.58	11.96	24.37	5.92	12.05

Pearl's[1] conclusion "that the size of eggs is not directly proportional to the number of yolks they contain." The data on which he based his conclusion were taken from the eggs of different individuals of various ages and the eggs were produced at different seasons of the year. Since all these factors influence the size and the proportion of parts of the egg it is interesting to note that his conclusion is confirmed by the present observation, made on eggs of a single individual laid in a period of three weeks' duration.

The data given in Table II. also show that the different parts of the double- and triple-yolked eggs are in different proportions than in single-yolked eggs. Fig 4 shows graphically for the eggs of 65K the mean weight of the egg and of each egg part for eggs with one, two and three yolks.

Both the diagram and the table show that the weight of albumen and shell are increased in double-yolked eggs. These components still further increase in triple-yolked eggs, but the increase in these parts is less than the increase in yolk weight. This may be seen either from the apparent slope of the lines in the figure, or from a comparison of the actual weight of the parts

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[1] *Loc. cit.*

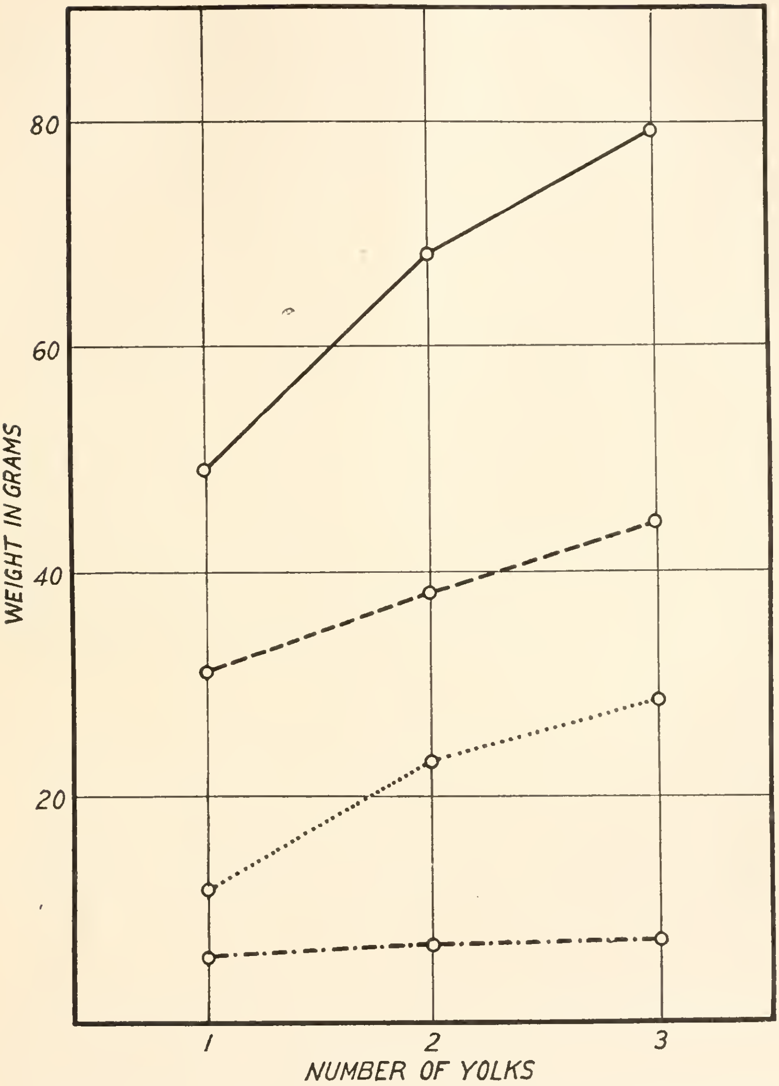

FIG. 4. Showing the mean weight in grams of the egg and each egg part for single-, double-, and triple-yolked eggs of 65K. Solid line = egg weight. Dash line = albumen weight. Dot line = yolk weight. Dash and dot line = shell weight.

in the different sorts of eggs, or from a comparison of the percentages. A comparison of the percentages shows that albumen forms 63.58 per cent. of the normal eggs of this bird, but only

56.07 per cent. of the double-yolked egg and only 55.56 per cent. of the triple-yolked egg. On the other hand the yolk, which forms only 24.37 per cent. of the normal egg, forms 33.91 per cent. of the double, and 35.52 per cent. of the triple-yolked egg. Finally the shell percentage decreases from 12.05 per cent. in eggs with one yolk, to 10.04 per cent. in eggs with two yolks, and 8.92 per cent. in the egg with three yolks.

The different relation of the egg parts in eggs with different numbers of yolks may also be seen by comparing the egg weight, or the weight of a part, in double- and triple-yolked eggs, with the weight of the egg or the same part of a single-yolked egg. If, as is usually assumed, the yolks of multiple-yolked eggs are of the same size as the yolks of normal eggs, the total yolk weight of double- and triple-yolked eggs will be respectively twice and three times the yolk weight of normal eggs. If in addition the weights of the other parts were directly proportional to the number of yolks contained, the weight of each part would be as many times the weight of the same part of the normal egg as there are yolks. That neither of the assumptions is correct for the eggs of 65K is shown by Table III.

TABLE III.

SHOWING FOR THE EGGS OF 65K THE RATIO THAT THE WEIGHT OF THE WHOLE OR OF EACH PART OF A MULTIPLE-YOLKED EGG IS OF THE WEIGHT OF THE WHOLE OR THE SAME PART OF A SINGLE-YOLKED EGG.[1]

No. of Yolks in the Egg.	Ratio of the Egg Weight to the Weight of a Single-yolked Egg.	Ratio of the Yolk weight to the Yolk weight of a Single-yolked Egg.	Ratio of the Albumen Weight to the Albumen Weight of a Single-yolked Egg.	Ratio of Shell Weight to the Shell Weight of a Single-yolked Egg.
2	1.38	1.93	1.22	1.16
3	1.62	2.36	1.42	1.20

The figures in this table as well as the actual weight figures in Table II. show that the yolks in the multiple-yolked eggs of this bird are smaller than those in the normal eggs. They also show that the weights of albumen and shell are not as many times the weight of the same parts in the normal egg as is the weight of the yolk. The fact that the yolks of the double-yolked eggs were smaller than the yolks of the single-yolked eggs, and that the yolks of the triple-yolked eggs were still smaller, is in this case probably due entirely to the fact that the multiple-yolked eggs were laid just as the bird was coming to sexual maturity and thus preceded practically all the normal eggs with which they are compared. The triple-yolked egg was the first egg laid by this young pullet and the double-yolked eggs were the fourth and seventh, while the single yolked eggs were the fifth and the eighth to sixteenth inclusive.

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[1] The mean of the ten single-yolked eggs given in Table II.

The effect of the maturity of the bird upon the size of the egg and especially upon the weight of the yolk has been discussed in a previous paper.[1] The question now arises as to whether in general the yolks of multiple-yolked eggs are smaller than the yolks of single-yolked eggs produced at the same period by the same individual.

In the course of the investigation referred to above some data bearing on this point were obtained. Five of the birds used in this investigation produced one or more double-yolked eggs. Data were taken on all of the eggs laid by each of these birds. It is thus possible to compare the actual size and the size and proportion of the parts of each double-yolked egg with the mean of the normal egg produced by the same bird at the same period. The mean was, therefore, calculated for the ten eggs which succeeded each double-yolked egg. These data are brought together in Table IV.

In this table data are given on eleven double-yolked eggs. Four of the eleven were either first or second eggs of a young pullet and are, therefore, of no value in the present connection. In all these, as would be expected, the weight of each yolk is less than the mean yolk weight of the succeeding eggs. Of the seven double-yolked eggs which were not first or second eggs, four have yolks which are practically the same size or larger, while three have yolks that are significantly smaller.

The size of each yolk of double-yolked eggs is probably in general not any smaller than that of the normal eggs produced by the same individual at the same period of production. When multiple-yolked eggs are among the first pullet eggs, however, the

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[1] Curtis, M. R., "A Biometrical Study of Egg Production in the Domestic Fowl, IV., Factors Influencing the Size, Shape and Physical Constitution of Eggs." (In press.)

TABLE IV.

The Dimensions, Shape, Weight and the Weight and Proportion of Parts of Each Double-Yolked Egg and the Mean of the Ten Succeeding Single-Yolked Eggs Produced by a Flock of Barred Plymouth Rock Birds During Their First Two Laying Years.

Bird No.	Egg No.	Date.	Length, Mm.	Breadth, Mm.	Index, Per Cent.	Weight of Egg, Gm.	Weight of Albumen, Gm.	Per Cent. Albumen.	Weight Yolk, Gm.	Per Cent. Yolk.	Weight Shell, Etc., Gm.	Per Cent. Shell.
218	7	Nov. 19, 1910.	65.60	43.61	66.48	73.27	40.84	55.74	12.73 11.78 24.51	33.45	7.91	10.81
218	Mean of 10 following eggs	....	51.78	39.42	75.87	45.95	27.86	60.62	12.63	27.49	5.47	11.90
218	239	Feb. 25, 1912.	70.90	46.30	65.30	87.20	47.58	54.56	17.04 15.01 32.05	36.75	7.57	8.68
218	Mean of 10 following eggs	....	60.00	42.01	70.06	59.04	33.19	56.18	19.81	33.58	6.04	10.22
218	342	Aug. 13, 1912.	76.20	46.36	60.84	92.71	48.32	52.12	18.64 18.57 37.21	40.14	7.18	7.74
218	Mean of 10 following eggs	....	57.20	41.16	72.03	54.07	31.19	57.69	17.46	32.29	5.42	10.03
218	350	Sept. 9, 1912.	74.30	47.70	64.20	91.97	46.21	50.24	19.34 18.97 38.31	41.65	7.45	8.10
218	Mean of 10 following eggs	....	59.34	42.06	70.96	57.58	32.88	57.11	18.92	32.85	5.78	10.04
218	Minimum for all normal 1st year eggs	....	......	......	67.40	......	......	......	......	......	......	......
139	1	Oct. 27, 1910.	71.77	42.15	58.73	77.41	45.10	58.26	11.51 12.26 23.77	30.71	8.54	11.03
139	Mean of 10 following eggs	....	54.38	40.34	74.22	50.49	30.00	59.37	14.37	28.50	6.12	12.13

TABLE IV.—*continued.*

Bird No.	Egg No.	Date.	Length, Mm.	Breadth, Mm.	Index, Per Cent.	Weight of Egg, Gm.	Weight of Albumen, Gm.	Per Cent. Albumen.	Weight Yolk, Gm.	Per Cent. Yolk.	Weight Shell, Etc., Gm.	Per Cent. Shell.
139	129	June 22, 1911.	71.80	44.10	61.42	80.21	45.05	56.17	14.11 13.91 28.02	34.93	7.14	8.90
139	Mean of 10 following eggs..................	....	57.87	41.90	72.43	57.17	35.36	61.83	16.28	28.49	5.53	9.68
139	160	Aug. 17, 1911.	68.00	45.75	67.28	85.97	47.35	55.08	16.08 15.37 31.45	36.58	7.17	8.34
139	Mean of 10 following eggs..................	....	58.16	42.33	72.82	59.03	36.93	62.59	16.29	27.57	5.81	9.84
130	Minimum for all normal 1st year eggs...	.....			63.87							
478	1	Nov. 7, 1910.	69.10	44.03	63.72	74.85	48.79	65.18	9.30 9.64 18.94	25.30	7.12	9.51
478	2	Nov. 9, 1910.	64.70	44.12	68.20	72.29	43.35	59.97	10.57 10.80 21.37	29.56	7.57	10.47
478	Mean of 10 following eggs..................	....	52.78	41.22	78.19	51.30	34.08	66.34	11.58	22.66	5.65	11.01
478	Minimum for all normal 1st year eggs.......	....	......	......	69.26	......	......	...	......			
192	2	Nov. 5, 1910.	60.85	39.60	65.08	54.77	27.38	49.99	10.09 10.94 21.03	38.40	6.36	11.61
192	Mean of 10 following eggs..................	....	52.34	37.65	71.94	42.71	24.99	58.52	12.87	30.12	4.85	11.36
192	Minimum for all normal 1st year eggs........	.....		......	69.10	......	......					
204	179	Feb. 12, 1912.	72.50	44.92	61.96	84.10	45.85	54.52	15.41 16.42 31.83	37.85	6.42	7.63
204	Mean of 10 following eggs..................	....	60.67	41.19	67.90	58.89	34.43	58.44	18.68	31.72	5.79	9.84
204	Minimum for all normal 1st year eggs........	....	......	......	65.73	......	......	......	......	......	......	......

yolks are smaller than those of succeeding eggs since at this period the successive yolks are increasing in size.

Table IV. shows also that in all cases the absolute weight of both albumen and shell is higher in double-yolked eggs than in the mean of the next ten normal eggs, but in none of the double-yolked eggs observed was the percentage of either albumen or shell as high as in the succeeding normal eggs. These observations are in agreement with those on the eggs of 65K (see Table II.). They show that while an increase in the stimulation causes an increase in the amount of secretion the increase is not in direct proportion to the weight of the substance causing the stimulation.

This may be due to the fact that the effective stimulation is not directly proportional to the weight. That is, it may be due to the size of the area of contact, or to the time the stimulus is applied to each successive area of the secreting membrane. Or again it may be due to a deficiency of material available for the formation of the secretion, or of time necessary for its elaboration.

In this connection attention should be called to two facts noted in previous paragraphs, first that when the eggs have separate chalazal membranes the chalazæ of the second yolk are usually much less developed than the first, and second that when the eggs have separate thick albumen envelopes the one surrounding the second yolk is more viscous but forms a thinner layer. In such cases at least the two yolks represent successive stimulations rather than a single intensified stimulation, and the glands are evidently not in a condition to respond in the same degree to the second stimulation. There is, of course, the possibility that the difference in the quantity and quality of secretion in the two cases may in part be due to the fact that the second yolk is moved more rapidly than the first but this theory would not account for the smaller per cent. of albumen in double-yolked eggs where the two yolks have common albumen envelopes.

The processes which determine the quantitative relationship between the yolk and albumen and between yolk + albumen and shell are too complex for solution with our present knowledge. Yet it is certain that although practically doubling and tripling the weight of yolks does not cause a proportionate increase in the secretion of albumen and shell, nevertheless doubling causes an increase and tripling a still slightly greater increase. This shows that either the glands of the oviduct are not all completely discharged during the formation of a single-yolked, or even a double-yolked egg, or that under stimulation they elaborate their secretions with great rapidity.

A Comparison between the Shape of Single- and Multiple-Yolked Eggs of the Same Individual.

It may be seen from Tables II. and IV. that there is a decided difference in the shape of single- and multiple-yolked eggs produced by the same bird. The multiple-yolked eggs are both longer and broader than the single-yolked eggs, but they are not as broad in proportion to their length. This may be best seen by comparing the length-breadth index $\frac{100 \text{ breadth}}{\text{length}}$ of the double-yolked or triple-yolked egg with the mean index for the ten succeeding eggs. In all cases the mean index for the normal eggs is higher, showing that the normal egg is broader in proportion to its length. In Table IV. the lowest index for the eggs produced by each bird during the first year is also given. In every case but one the index of the double-yolked egg is lower than the minimum for all the first year normal eggs. That is, it falls below the normal variation for the individual. The multiple-yolked egg then is longer in proportion to its breadth than any of the normal eggs of the same individual.

The yolks pass down the duct in succession, and not abreast. In the complete egg they lie with their common axis in the long axis of the egg. They must, therefore, stimulate the oviduct glands in succession. However, the eggs with two or three yolks are actually broader than those with one. This may be due in part to the fact that the field of stimulation of the two yolks overlaps, but probably the most important factor is the mechanical one due to forcing (by peristalsis) a larger plastic mass through an elastic tube, which offers more resistance to the passage of a large than a small body.

Summary.

1. During the last six years more than three thousand different domestic fowls, which have been kept at least one year at the Maine Agricultural Experiment Station, have laid but three triple-yolked eggs.

2. Each of these eggs was laid by a different individual and in each case the triple-yolked egg was one of the first eggs produced by a young pullet.

3. Young pullets also show a decided tendency to produce double-yolked eggs when they first begin to lay. About 20 per cent. of the pullets which lay before they are seven months old lay among their first eggs one or more with two yolks.

4. Nearly 80 per cent. of the individuals of the flock never lay a double-yolked egg.

5. Mature birds also sometimes produce double-yolked eggs; but most such birds have also produced one or more when they were young pullets.

6. There has been no bird in the experiment station flock with which the laying of double-yolked eggs was "habitual" although there are some which have produced several such eggs.

7. The production of an egg with two or three yolks represents the extreme of rapid egg production, other forms of which are found in the production of two eggs united by a membranous tube; two eggs at the same time; two eggs at different times on the same day and a daily egg production where the eggs are laid earlier on each successive day.

8. The two yolks of a double-yolked egg may have all the egg envelopes in common, indicating that they have passed the entire length of the duct together; or each may possess one or more separate envelopes. There are also all the possible intermediate forms indicating that the two yolks in a common shell may unite at any point between the mouth of the funnel and the isthmus. When two eggs come together after the first has entirely passed the anterior end of the isthmus the result is the production of two eggs at the same time.

9. Various disturbances of the normal processes of egg production may bring two yolks together in the oviduct. Double-yolked eggs evidently do not always represent simultaneous

ovulations. The assumption of simultaneity or abnormally close succession of ovulations is necessary to account for the production of a succession of double-yolked eggs or of a double-yolked egg immediately following a long series of normal daily eggs.

10. The double-yolked eggs contain more albumen and have a heavier shell than single-yolked eggs, and in triple-yolked eggs these parts are heavier than in double-yolked eggs. Yet these parts do not increase in direct proportion to the increase in the weight of yolk. That is, the percentage of albumen and shell is less in double- than in single-yolked eggs and is still smaller in triple-yolked eggs.

11. The yolks of the multiple-yolked eggs of mature birds are not consistently smaller than the yolks of the normal eggs produced during the same period.

12. Multiple-yolked eggs are longer in proportion to their breadth than the normal eggs of the same individual.

The physiological bearing of these facts is discussed.

THE CHANGE IN VOLUME OF ARBACIA AND ASTERIAS EGGS AT FERTILIZATION.[1]

OTTO GLASER.

In my paper on the initiation of development, Glaser (13), I stated that the eggs of *Arbacia punctulata* and *Asterias forbesii* are smaller in volume after fertilization than before. In the pages that follow I wish to present the evidence for this assertion in some detail, since for the present at least, it must stand alone. *Strongylocentrotus* (*purpuratus?*) shows no recognizable loss of volume according to Loeb ('08) but this result does not contradict mine. The loss depends on several conditions, and in *Asterias* is much greater than in *Arbacia*. This suggests the possibility of forms in which it may easily be too small to measure. The measurements of McClendon ('10), also, do not contradict mine, although they seem to show that the very eggs I worked with, on fertilization lose in a molar solution of dextrose, but remain constant, or even gain a little in sea water. The evidence consists in the determination of the diameters of 19, 20, 10 and 11 eggs respectively. The great variability of the ova of *Arbacia punctulata*, however, proves that reliable results can be gotten from a small number of eggs, only when the identical ones are measured before and after fertilization.

Careful tracings were made at known magnifications with the aid of a camera lucida, and a crow-quill pen, on paper of known weight. On the assumption that the eggs are spheres, and that one is really drawing their great circles, the areas of the outlines obtained in this manner, were first determined by means of a planimeter, and afterwards by carefully cutting them out and weighing the discs. For enlargements of 740 diameters both methods gave identical results, but for the smaller magnifications the method of weighing proved more reliable. For this reason, in the comparisons of fertilized with unfertilized eggs, only data obtained by the latter method are reported, whereas the variability curves are based on factored measurements by the planimeter. The same curves were first constructed from the original measurements.

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[1] From the Marine Biological Laboratory at Woods Hole, and Zoölogical Laboratory of the University of Michigan.

ON THE CONSTANCY IN VOLUME OF THE EGGS OF A SINGLE FEMALE.

In Table I. are given the diameters of 280 different eggs derived from one female, measured in groups of ten, at three distinct periods after removal from the ovaries. The results show not only a remarkable constancy for the ova of a single individual, when grouped in this manner, but what is of even more immediate importance, they show that in four hours, the eggs do not undergo any change in volume discoverable by the methods employed.

TABLE I.

FEMALE B.

10 eggs, 4.35 P.M.	81.4 micra.	5.40 P.M.	82.6 micra.	8.35 P.M.	79.6 micra.
10 eggs, 4.35 P.M.	80.0 micra.	5.40 P.M.	80.8 micra.	8.35 P.M.	81.6 micra.
10 eggs, 4.35 P.M.	81.2 micra.	5.40 P.M.	80.8 micra.	8.35 P.M.	79.6 micra.
10 eggs, 4.35 P.M.	80.6 micra.	5.40 P.M.	79.4 micra.	8.35 P.M.	79.0 micra.
10 eggs, 4.35 P.M.	82.4 micra.	5.40 P.M.	78.6 micra.	8.35 P.M.	81.0 micra.
10 eggs, 4.35 P.M.	80.6 micra.	5.40 P.M.	81.6 micra.	8.35 P.M.	82.4 micra.
10 eggs, 4.35 P.M.	78.8 micra.	5.40 P.M.	81.2 micra.	8.35 P.M.	82.2 micra.
10 eggs, 4.35 P.M.	81.4 micra.	5.40 P.M.	80.8 micra.	8.35 P.M.	81.0 micra.
10 eggs, 4.35 P.M.	78.2 micra.	5.40 P.M.	77.4 micra.	8.35 P.M.	80.0 micra.
10 eggs, 4.35 P.M.	83.6 micra.	5.40 P.M.	micra.	8.35 P.M.	micra.
Average	80.8 micra.	Average	80.4 micra.	Average	80.6 micra.

ON THE VOLUME OF THE EGGS OF DIFFERENT FEMALES.

To what extent the average volume of the ova of different females is constant, I am unable to say, since scarcely anything much less than one hundred eggs from each would suffice to decide the question. One would not expect great constancy however unless the numerous factors entering into the result were identical. In the one case in which I have the requisite number of measurements for such comparison, the average diameter is the same as that found for female B.

Table II.

Female C.

10 eggs 80.4 diam.
10 eggs 82.2 diam.
10 eggs 80.2 diam.
10 eggs 79.6 diam.
10 eggs 81.6 diam.
10 eggs 79.0 diam.
10 eggs 77.8 diam.
10 eggs 79.2 diam.
10 eggs 79.2 diam.
Average 80.0 diam.

The Diameters of Individual Eggs.

In the two preceding tables the measurements are given for groups of ten eggs each. Comparison of the various sets indicates considerable variability, but the true extent of this can only be realized when a large number of ova is treated individually. This was done with 100 eggs of female B and 100 of female C. The results are given in Tables III. and IV.

Table III.

Female B, Individual Eggs.

Of 100 eggs	1 had a diameter of	49.8 micra.	
Of 100 eggs	1 had a diameter of	58.8 micra.	Average of 3 = 57.1
Of 100 eggs	1 had a diameter of	61.6 micra.	
Of 100 eggs	1 had a diameter of	64.2 micra.	
Of 100 eggs	1 had a diameter of	65.4 micra.	Average of 3 = 65.5
Of 100 eggs	1 had a diameter of	66.8 micra.	
Of 100 eggs	2 had a diameter of	68.0 micra.	
Of 100 eggs	4 had a diameter of	70.6 micra.	Average of 11 = 70.8
Of 100 eggs	5 had a diameter of	72.0 micra.	
Of 100 eggs	5 had a diameter of	73.2 micra.	
Of 100 eggs	3 had a diameter of	74.6 micra.	Average of 16 = 74.2
Of 100 eggs	8 had a diameter of	76.0 micra.	
Of 100 eggs	3 had a diameter of	77.2 micra.	
Of 100 eggs	5 had a diameter of	79.6 micra.	Average of 15 = 79.2
Of 100 eggs	7 had a diameter of	79.8 micra.	
Of 100 eggs	8 had a diameter of	81.2 micra.	
Of 100 eggs	2 had a diameter of	82.4 micra.	Average of 20 = 82.6
Of 100 eggs	10 had a diameter of	83.8 micra.	
Of 100 eggs	7 had a diameter of	85.0 micra.	
Of 100 eggs	4 had a diameter of	86.4 micra.	Average of 14 = 84.5
Of 100 eggs	3 had a diameter of	87.6 micra.	
Of 100 eggs	6 had a diameter of	89.0 micra.	

TABLE III. *Continued.*

FEMALE B. INDIVIDUAL EGGS.

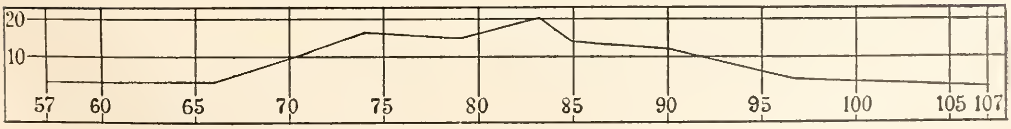
Of 100 eggs 3 had a diameter of 90.4 micra.	Average of 12 = 90.0
Of 100 eggs 3 had a diameter of 91.6 micra.	
Of 100 eggs 2 had a diameter of 94.2 micra.	
Of 100 eggs 1 had a diameter of 96.8 micra.	Average of 4 = 96.5
Of 100 eggs 1 had a diameter of 100.8 micra.	
Of 100 eggs 1 had a diameter of 102.0 micra.	
Of 100 eggs 1 had a diameter of 112.6 micra.	Average of 2 = 107.3

TABLE IV.

FEMALE C, INDIVIDUAL EGGS.

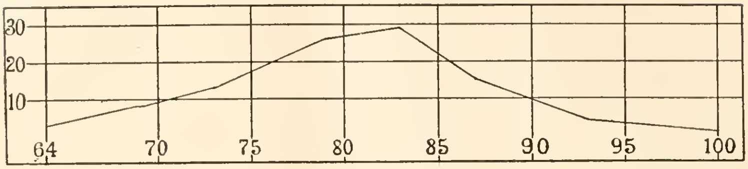
Of 100 eggs 1 had a diameter of 59.4 micra.	
Of 100 eggs 1 had a diameter of 65.2 micra.	Average of 3 = 63.7
Of 100 eggs 1 had a diameter of 66.6 micra.	
Of 100 eggs 2 had a diameter of 68.0 micra.	
Of 100 eggs 5 had a diameter of 69.6 micra.	Average of 8 = 69.4
Of 100 eggs 1 had a diameter of 71.0 micra.	
Of 100 eggs 5 had a diameter of 72.2 micra.	
Of 100 eggs 4 had a diameter of 73.8 micra.	Average of 13 = 72.9
Of 100 eggs 4 had a diameter of 75.4 micra.	
Of 100 eggs 6 had a diameter of 76.8 micra.	
Of 100 eggs 9 had a diameter of 78.2 micra.	Average of 26 = 78.5
Of 100 eggs 11 had a diameter of 79.6 micra.	
Of 100 eggs 8 had a diameter of 81.0 micra.	
Of 100 eggs 11 had a diameter of 82.6 micra.	Average of 29 = 82.6
Of 100 eggs 10 had a diameter of 83.8 micra.	
Of 100 eggs 7 had a diameter of 85.6 micra.	
Of 100 eggs 4 had a diameter of 87.0 micra.	Average of 15 = 86.7
Of 100 eggs 4 had a diameter of 88.4 micra.	
Of 100 eggs 2 had a diameter of 89.8 micra.	
Of 100 eggs 1 had a diameter of 94.2 micra.	Average of 4 = 92.4
Of 100 eggs 1 had a diameter of 95.6 micra.	
Of 100 eggs 1 had a diameter of 100.0 micra.	Average of 1 = 100.0

If one treats the diameters and eggs in groups of three, and plots the latter on the ordinate and the former on the abscissa the following curve emerges for female B.



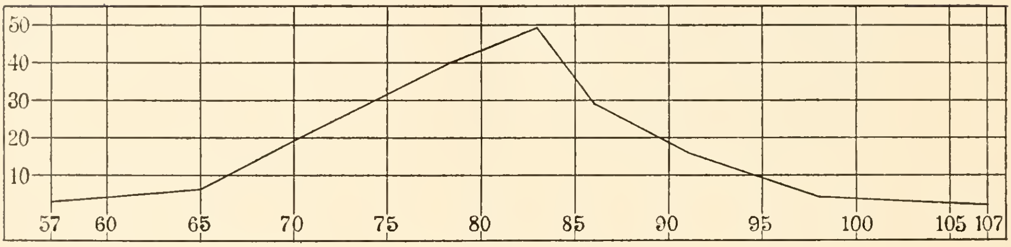
CURVE B. Diameters of 100 eggs from one specimen of *Arbacia punctulata*.

The data from female C, treated similarly, give curve C.



Curve C. Diameters of 100 eggs from one specimen of *Arbacia punctulata*.

Since the diameters for females B and C on the average are equal, the eggs of these two may be considered as homogeneous material. If this is done, then after the usual statistical simplifications,[1] an almost symmetrical curve results.



Curve BC. A composite of the diameters of 200 eggs from two specimens of *Arbacia punctulata*.

Comparison of Unfertilized and Fertilized Ova.

The variability exhibited by these eggs and the differences in range between different females, show clearly that if one wishes to make trustworthy comparisons between the volumes of unfertilized and fertilized ova, it is either necessary to use a large number of each kind from a single female or to deal with a smaller number and measure the identical eggs before and after fertilization.

Before adopting the second alternative, I made a series of tracings of unfertilized and fertilized ova to see whether measureable differences really existed. The results, averaged, were as follows:

Unfertilized	eggs; 77 measurements;	average diameter,	74.1 micra.
Fertilized	eggs; 91 measurements;	average diameter,	70.7 micra.
		Loss	3.4 micra.

---

[1] The eggs are treated in sets of three, and average diameters not more than three micra apart are considered as belonging to the same group.

This result, further discussion of which is unnecessary, encouraged me to undertake a more careful study of a few eggs. Although involving more labor, this alternative was chosen in preference to the "wholesale" method, because the behavior of individual eggs could be definitely recorded. The probable advantage of this was indicated by the discovery by Glaser ('13) that the number of sperm used in insemination plays a rôle in the result. Accordingly "wholesale" measurements, which are satisfactory enough to decide whether or not a determinable change in volume occurs, have the disadvantage of covering up the fact that the loss is by no means a constant quantity.

In making these particular measurements, special precautions were taken. The eggs were traced one at a time, no other eggs were on the slide, and the focus before and after fertilization remained unchanged. This was not easy to accomplish, for the sperm are apt to set the egg in motion and change its position. Whenever this occurred, the attempt to secure the second measurement was abandoned. Naturally only a small number of cases could be collected under these conditions.

In order to fulfill the aforesaid requirements, and keep the magnification of the tracings absolutely constant, it was necessary to use small quantities of sperm. This however introduced other difficulties. The appearance of the fertilization membrane is the only convenient index of impregnation, but the membrane does not appear unless the spermatozoa are present in sufficient numbers. As a consequence, I often failed to observe the only available indication of fertilization.

While the results which I have seem to me to point in but one direction, the changes in volumes registered are in most cases probably minimal, for they depend on the amount of surface alteration, and this in turn is, within limits, a function of the number of sperm involved. Indeed with great excesses of sperm I have not infrequently observed a destructive alteration of the surface of the egg. This is more easily observable in *Asterias* than in *Arbacia*. It appears likely therefore that normally, or perhaps better, frequently, greater changes in volume occur than I have recorded.

The results on individual eggs dealt with singly in the manner described are given in the subjoined table.

Table V.

Individual Eggs Before and After Fertilization.

Female.	Egg.	Measurements.	Before.	Measurements.	After.	Change in diameter.
D_1	1	3	81.2	2	75.4	−5.8
D_1	2	3	80.6	3	68.8	−11.8
D_1	3	3	73.4	3	65.4	−8.0
D_1	4	3	78.8	3	72.8	−6.0
D_1	5	3	74.6	3	68.4	−6.2
D_2	1	4	70.0	3	66.6	−3.4
D_2	2	4	71.2	4	67.2	−4.0
D_2	3*	3	78.0	4	78.2	+0.2*
D_2	4	4	75.2	3	73.4	−1.8
D_2	5	3	78.0	4	75.2	−2.8
E	1	3	70.0	3	68.2	−1.8
E	2*	4	65.2	2	65.8	+0.6*
E	3	3	72.2	4	65.6	−6.6
E	4	3	72.2	4	69.4	−2.8
E	5	3	68.8	4	67.8	−1.0

With the exception of the starred eggs, D_2 3, and E 2, which within the limits of error may easily be considered constant, all the eggs showed a noticeable decrease.

The fact brought out by these measurements constitutes an additional support for the conception that an increase in the permeability of the egg accompanies fertilization. With such increase, other conditions remaining constant, one would expect a change in volume. The fact that unfertilized eggs, under certain conditions, may remain unaltered for hours, whereas there is a noticeable decrease in their diameters immediately after fertilization, strongly emphasizes the change that takes place.

Comparison of Unfertilized and Fertilized Ova of *Asterias*.

The results obtained in the case of *Arbacia* are more strikingly illustrated by a control observation made on the ova of *Asterias forbesii*. The measurements were made on eggs which had been removed from the ovaries and allowed to stand one hour in sea water in order to complete maturation. Tracings were made of two small groups from two females and these groups were then inseminated. It is impossible to identify individual eggs in this case, but this does not invalidate the result, since no eggs were either added or removed upon the addition of the sperm. Seventeen eggs derived from one female, and eight from the other were used, and the same eggs were traced after fertilization that had been traced before. The result was:

Female 1; 17 eggs	Diameter before fertilization, 103.6 micra. Diameter after fertilization, 85.2 micra.	Loss 18.4 micra.
Female 2: 8 eggs	Diameter before fertilization, 105.0 micra. Diameter after fertilization, 94.0 micra.	Loss 11.0 micra.

These values cannot be considered standard figures any more than those found in the case of the sea urchin, yet they seem to indicate with certainty that the egg of the starfish also loses in volume on fertilization. In fact the loss recorded is much greater than that found for *Arbacia* eggs. A portion of this difference may be due to unequal amounts of sperm used in the two cases, but the entire variation can hardly be accounted for on this basis. Normally the perivitelline space of fertilized *Asterias* eggs is proportionately larger than in *Arbacia*. If the space in question comes about as Loeb ('08) has contended, when a colloidal substance secreted by the egg absorbs water, then on the assumption that this material has essentially the same properties in *Arbacia* and in *Asterias*, the egg having the greater vitelline space should secrete the greater quantity, and therefore decrease correspondingly more in volume. That the *Asterias* eggs measured lost more on the average than the *Arbacia* treated either en masse, or as individuals, can be seen by a glance at the tables. One is led to suspect from these considerations that the surface of the *Asterias* egg is more easily modifiable than that of the *Arbacia* ovum. This is in harmony with the well known experience that artificial parthenogenesis can be induced in *Asterias* by a greater variety of methods than in *Arbacia*.

UNIVERSITY OF MICHIGAN,
November 7, 1913.

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VOLUMETRIC CHANGES IN THE EGG OF THE BROOK LAMPREY, ENTOSPHENUS (LAMPETRA) WILDERI (GAGE), AFTER FERTILIZATION.[1]

PETER OKKELBERG.

Various investigators have found by actual measurements that the eggs of certain animals do not change appreciably in volume as a result of fertilization. Calberla[2] in his work on the fertilization process in the egg of *Petromyzon planeri* says: "Genaue Messungen mittelst des Zeichnenprismas (Oberhäuser), die während des Befruchtungsvorganges angestellt wurden, erwiesen es als zweifellos, dass der Dotter sich nicht contrahirt, dagegen die Eihaut sich enorm ausdehnt. . . . Allerdings nimmt der Dotter, nachdem er von der Eihautberührung befreit worden ist, eine andere Form an, d. h. er geht von der Ellipsoidform in die Kugelform über, aber dies geschieht ohne Volumsverminderung." McClendon,[3] working on the eggs of *Arbacia punctulata*, found that when the eggs were placed in a molecular solution of cane sugar, approximately isosmotic with sea water, the mean diameter of a certain number of eggs was 83 when not fertilized and 75 when fertilized. Control eggs in ordinary sea water showed mean diameters of 83 and 86 respectively when unfertilized and fertilized. According to these results the eggs of *Arbacia punctulata* do not decrease in size as a result of fertilization under normal conditions but become larger instead. Loeb[4] found that the volume of the egg of *Strongylocentrotus* does not change appreciably as a result of fertilization. He says: "Ich habe eine Reihe von Messungen der Durchmessers des Cytoplasmas vor und nach der Befruchtung mit Hilfe des Zeichenapparates ausgefuhrt, wohin es sich herausstellte, dass das Volumen des Eicytoplasmas von *Strongylocentrotus* bei der

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[1] Contribution from the Zoölogical Laboratory of the University of Michigan, No. 141.

[2] Calberla, Ernst, *Zeitschr. f. wiss. Zool.*, Vol. 30, 1877.

[3] McClendon, J. F., *Science*, N.S., Vol. 32, 1910.

[4] Loeb, J., *Arch. f. Entwicklungsmechanik*, Vol. 26, 1908.

Membranbildung keine mit dieser Methode wahrnehmbare Volumänderung erfährt. Das beweist mit Sicherheit, dass der Inhalt des Membranraums hauptsächlich Seewasser ist, das vom aussen in demselben diffundiert."

In some work that I have done[1] on the fertilization process in the brook lamprey, *Entosphenus wilderi* (Gage), I have obtained results which indicate that the eggs in this form decrease considerably in size after fertilization.

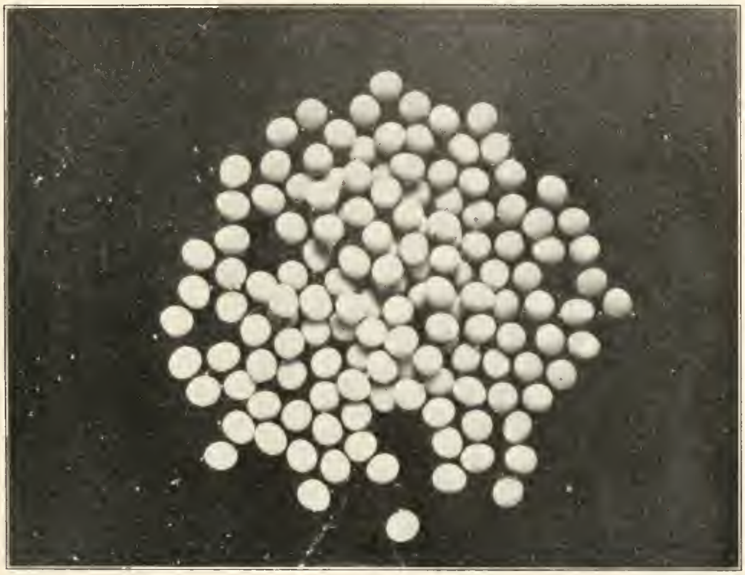

FIG. 1. Unfertilized eggs of *Entosphenus wilderi.*

Herfort[2] has given a careful description of the egg of *Petromyzon fluviatilis* and I have verified most of his observations on the egg of the American brook lamprey. Since there is no essential difference between the eggs of the two species, only a brief description is necessary here. Before fertilization the egg approaches very closely to an ellipsoid of revolution in shape although it is slightly more pointed at the animal pole (Fig. 1). It possesses a distinct membrane which has been termed by Lubosch[1] and others the zona radiata but to which I shall apply the more general term vitelline membrane. In the ovarian egg of the larva this membrane consists of a single layer but after metamorphosis it becomes differentiated into two distinct layers which increase in thickness as the egg approaches maturity. The outer layer usually shows faint striations which have been supposed to represent canals. The two layers are very firmly united. Beneath the vitelline membrane there is a cortical alveolar layer which is covered on the outside by a very thin protoplasmic layer (vitelline membrane of Lubosch). This thin protoplasmic layer adheres very closely to the vitelline membrane and is continuous with the rest of the egg protoplasm through the walls of the alveoli. The alveolar layer seems to be similar to that in *Nereis* as described by F. R. Lillie.[2] The same condition has also been described by Reighard[3] in the egg of the wall-eyed pike. I have not been able to find any perivitelline space in the unfertilized egg.

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[1] Presented to the Michigan Academy of Science at its nineteenth annual meeting in Ann Arbor, April 3, 1913. Fifteenth Report of the Michigan Academy of Science.

[2] Herfort, Karl, *Arch. f. mikr. Anat.*, Vol. 57, 1901.

Outside of the vitelline membrane there is a gelatinous substance which becomes adhesive upon the addition of water. This substance is supposed by Böhm[4] and others to be derived from the follicular epithelium of the ovarian egg. It is thickest at the vegetative pole and thins off gradually towards the animal pole. Here it is covered by a mucous mass which was first described by A. Müller[5] who called it the "Flocke." It is in the "Flocke" that most of the spermatozoa are found after the egg is fertilized. The vitelline membrane is slightly modified at the animal pole to form a region permeable to the spermatozoa but there appears to be no true micropyle present.

After fertilization visible changes almost immediately take place which result in the separation of the vitelline membrane from the egg and the formation of a large perivitelline space (Fig. 2). This separation is initiated a short distance from the middle of the animal pole as an indentation of the cortical layer, and a circular perivitelline space is formed which extends around the pole in the form of a groove. This groove widens rapidly towards the animal pole until it forms a large space separating the egg at this pole from the vitelline membrane. At the same time a wave of contraction passes over the egg towards the vegetative pole and within a few minutes the whole egg is separated from the membrane. During the contractions of the egg, the long and short diameters vary considerably from time to time. I have made accurate measurements at short intervals which show that the contractions are real and not merely apparent. They are very striking and remind one of peristaltic waves or ameboid movements.

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[1] Lubosch, W., *Jen. Zeitschr. f. Naturwiss.*, Vol. 38, 1904.

[2] Lillie, F. R., *Jour. Morph.*, Vol. 22, 1911.

[3] Reighard, Jacob, Tenth Biennial Report of the State Board of Fish Commissioners (Michigan), 1893.

[4] Böhm, A. A., *Arch. f. Mikr. Anat.*, Vol. 32, 1888.

[5] Müller, A., Herrn Karl von Baer zur Feier des 50. Jahrestages, u.s.w., 1864.

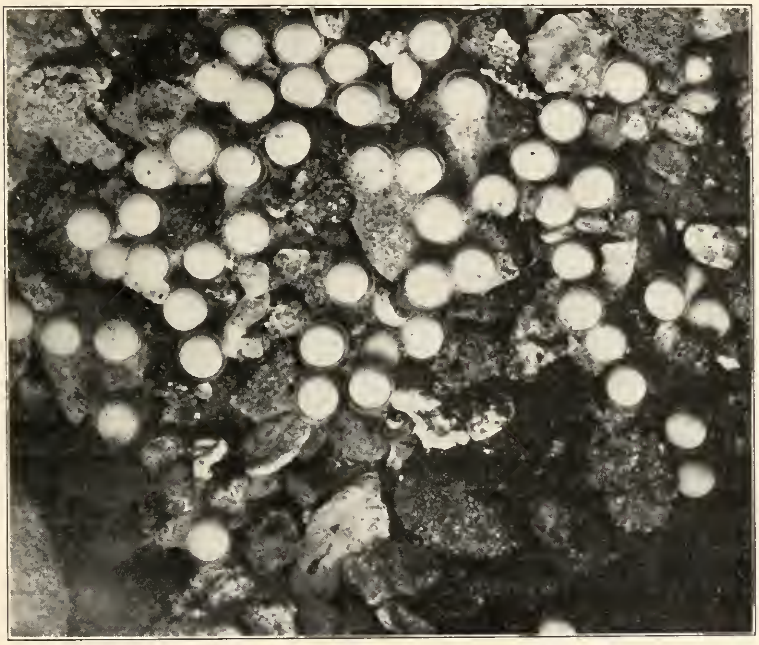

FIG. 2. Fertilized eggs of *Entosphenus wilderi*. ×6.

The whole process during which the membrane separates from the egg lasts about five minutes and when the egg comes to rest again it has assumed a spherical shape instead of the previous ellipsoidal or ovoidal shape. During the process the vitelline membrane becomes greatly distended. When the membrane first begins to separate from the egg, the walls of the alveoli of the cortical layer are drawn out into long slender threads which soon break, the outer part adhering to the vitelline membrane and the inner part fusing with the cytoplasm of the surface of the egg. The contents of the alveoli do not seem to diffuse through the vitelline membrane but remain in the perivitelline space. The thin layer of cytoplasm which adheres to the vitelline membrane seems to be similar to the same layer found in the fertilized egg of *Nereis*. In this form F. R. Lillie regards it as a plasma membrane "comparable in some respects to the fertilization membrane of the sea urchin" (loc. cit., page 364). This thin film does not seem to be a permanent structure in the lamprey egg and probably breaks up as the vitelline membrane distends. A part of it, at least, together with the cytoplasm of the alveolar walls seems to collect into little drops which float off into the fluid of the perivitelline space and eventually disintegrate.

Without going into any further detail concerning the structure of the egg and the changes it undergoes as a result of fertilization, we may now return to our problem of determining the relative size of the egg before and after fertilization.

The egg of the brook lamprey is noticeably smaller after fertilization than before. From careful measurements the volume of the egg before and after fertilization may be calculated with some degree of accuracy and the decrease in volume thus ascertained. In the case of an egg that is spherical in shape both before and after fertilization, as for instance the sea urchin egg, the diameter alone is the only dimension needed in order to calculate the volume. For the unfertilized lamprey eggs a different method must be employed. For purposes of calculation they were considered as perfect ellipsoids of revolution. This introduces an error but I am convinced it can not be very great, as the eggs are very nearly ellipsoidal in shape.

The unfertilized eggs were placed in a watch glass containing ordinary tap water at a temperature of about 19 degrees Centigrade and outlined by means of a camera lucida at a magnification of 80 diameters. These outlines were as nearly as possible optical sections through the plane of the major axis of the eggs and did not include the vitelline membrane. The eggs were then fertilized and after about ten minutes they were outlined again in a similar manner through the planes of their diameters. The drawings thus obtained were then measured by means of a planimeter which recorded the area in square centimeters. From these areas and the long diameters in the case of the unfertilized eggs, and the areas and the diameters in case of the fertilized eggs, the volumes were calculated according to formulas as follows: The volume of an ellipsoid of revolution is equal to the constant .8488 times the square of the area of the plane of its major axis divided by the major axis; and the volume of a sphere is equal to the constant .8488 times the square of the area of its great circle divided by the diameter.[1] Formulas involving the use of planimeter areas and diameters were used because they seemed to introduce the least number of factors and thus eliminate chances of error. Since the same factors were used in each case the errors ought to average up about the same for both sets of drawings.

The volumes of 48 eggs at a magnification of 80 diameters were thus ascertained before and after fertilization. The volumes thus obtained were divided by the cube of 80 in order to get the actual volumes of the eggs. This gave as a result an average volume of .6017 cubic millimeter for the unfertilized eggs and an average volume of .5205 cubic millimeter for the fertilized eggs. This shows that the average decrease in size is .0812 cubic millimeter or 13.48 per cent. of the original volume.[2]

The above results are so striking that they have seemed worth recording at this time. Analogous conditions have been found by O. C. Glaser[3] in the egg of *Asterias forbesii*. Here the egg separates after fertilization from the inner surface of a thin preëxisting membrane and the change does not seem to be due to changes in the fertilization membrane but to changes in the surface film of the egg. The egg of *Asterias* after separating from the membrane was found to be smaller than before. In case of the lamprey egg also the initial change seems to be in the cortical layer of the egg and not in the membrane.

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[1] I am indebted to Professor Theodore R. Running for suggesting the use of these formulas.

[2] Professor Reighard has called my attention to the fact that since the unfertilized eggs are heavier than the water in which they are kept and rest on a plane surface, they may be slightly flattened by gravity. The fertilized eggs, on the other hand, which are immersed in a perivitelline fluid of greater density than water and rest on the curved vitelline membrane, are less likely to be flattened by gravity. For these reasons my measurements for the unfertilized eggs might be larger than those for the fertilized. I have examined unfertilized eggs that have been fixed in various fixing solutions but have found no evidence of flattening in these. There is no fresh material available at present. Since the eggs are small and were kept in water when they were outlined it does not seem probable that they would flatten to any appreciable extent.

[3] Glaser, O. C., *Science*, N.S., Vol. 38, 1913.

I have succeeded in inducing the egg of the lamprey to go through changes similar to those it exhibits after fertilization, by subjecting it to artificial stimuli, such as shaking, pricking with a needle, exposing to sudden changes of temperature, etc. The membrane separates in a similar manner f om the egg, a peristaltic wave passes over it and it becomes spherical in shape. This indicates that the stimulus that starts the egg on a career of development need not be of any specific kind. Some of the lamprey eggs that have thus been stimulated have segmented parthenogenetically. Bataillon[1] has succeeded in inducing parthenogenetic development of the egg of *Petromyzon planeri* by introducing them into a solution of cane sugar, and he has demonstrated that the segmentation is real and not merely a fragmentation of the egg.

Most of the material that the eggs lose as a result of fertilization is probably water, but it seems to be certain that other substances are also given off. Reighard (loc. cit., page 105) called the fluid contents of the alveoli of the cortical layer in the egg of the wall-eyed pike "cortical drops" and came to the conclusion that these drops consisted of an albuminous substance and that this substance is in solution in the perivitelline fluid. F. R. Lillie (loc. cit., page 365) thinks that the contents of the cortical alveoli in the egg of *Nereis* are unquestionably colloidal. Similar conclusions have been reached by others in other forms. In the lamprey egg after fertilization much of the substance that fills up the space between the egg and the membrane is water that has diffused through from the outside. Some of the substance, however, came from the cortical alveoli and is probably a colloid. F. R. Lillie[2] has found that the unfertilized ova of *Nereis* and *Arbacia* in sea water secrete substances which cause agglutination of their respective spermatozoa. He has termed this substance "fertilizin" and assumes that "the union of this substance with the spermatozoa enters in some significant way into the process of development," and that it is located in the cortex of the egg. It may be that the "Flocke" described for the lamprey egg has some significance in this connection for it is in it that the spermatozoa seem to collect in great numbers after fertilization.

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[1] Bataillon, E., *Arch. f. Entwicklungsmechanik*, Vol. 18, 1904.

[2] Lillie, F. R., *Science*, N.S., Vol. 38, 1913.

Evidences presented in this paper show clearly that the egg of the brook lamprey decreases considerably in size after fertilization. This is contrary to the results obtained by Calberla in the egg of *Petromyzon planeri*, by McClendon in *Arbacia punctulata*, and by Loeb in *Strongylocentrotus*. It harmonizes, however, with the results obtained by Glaser in *Asterias forbesii*. There is reason for believing that some of the substance given off by the egg after fertilization is colloidal in nature. Much of this substance comes from the cortical alveoli.

I desire to express my thanks to Professor Jacob Reighard for his criticism in connection with the preparation of this paper.

Ann Arbor, Mich.,
November 17, 1913.

Vol. XXVI. *March, 1914.* *No. 3*

BIOLOGICAL BULLETIN

THE GASTRIC CÆCA AND THE CÆCAL BACTERIA OF THE HETEROPTERA.

HUGH GLASGOW.

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SUMMARY.

1. Certain of the Heteroptera are almost unique among insects in the possession of well-developed cæcal appendages situated at the extreme posterior end of the digestive portion of the gut.

2. The cæca are remarkable in that wherever they occur they are invariably filled with bacteria.

3. These bacteria are not only always present in the cæca of normal bugs but the association is hereditary, the organisms appearing early in the alimentary canal of the developing embryo.

4. Structurally the bacteria from different hosts vary greatly, ranging from minute, coccus-like bacilli measuring often less than one micron, to huge, spirochæte-like forms thirty microns and more in length; but in whatever form they occur they are morphologically characteristic for the particular species harboring them.

5. That these are unquestionably bacteria is shown conclusively by culture experiments checked by agglutination tests.

6. These normal bacteria appear not only to inhibit the development of foreign bacteria but to exclude them altogether,

the mid-intestine being normally wholly free from the invading bacteria and protozoa which are common in many related insects· and this is probably the chief function in the life processess of the host performed by these cæcal bacteria.

7. The cæcal appendages themselves appear to be of profound phylogenetic significance, showing a complete gradation from extremely simple to very complex forms and in many cases indicate relationships contrary to those often assumed in the arrangement of the groups.

Introduction.

In certain groups of Heteroptera the alimentary canal is characterized by the presence of peculiar sac-like appendages which open into the mid-intestine at its extreme posterior end. These structures vary greatly in form and in degree of development in the different families in which they occur, but all have essentially the same histological structure and all agree in the fact that they invariably contain great masses of bacteria, apparently in pure culture, which are morphologically characteristic for the families and often for the genera in which they occur.

These structures were noted as early as 1809 by Treviranus in a pentatomid, *Pentatoma rufipes* (*Cimex rufipes*), and again in 1811 by Ramdohr in *Pyrrhocoris apterus* as well as in representatives of the Pentatomidæ, and they were subsequently observed and studied by numerous other investigators, notably by Dufour in 1833. It was not until about 1888, however, that Professor S. A. Forbes (Forbes, '96), in the course of an elaborate series of investigations on the contagious diseases of insects, discovered the remarkable association existing between certain Heteroptera and the bacteria which he found uniformly inhabiting the so-called cæcal glands. Leydig had previously noted the presence of these organisms in the cæcal appendages of a pentatomid in 1857, but he did not suggest their true nature and merely expressed surprise at the occurrence of such unusual structures in these organs.

The cæcal bacteria were really first observed by Professor Forbes in 1882 in crushed specimens of the chinch bug, *Blissus leucopterus*, and a brief technical description of the organism, under the name *Micrococcus insectorum*, was given it by Professor

T. J. Burrill in 1883. The specimens first examined by Professor Forbes were from experimental cages of potted corn in which the insects had been dying in large numbers from some cause which even at the present time is not clearly understood, and upon finding large numbers of specific bacteria in crushed preparations of these insects he at first very naturally regarded them as the probable cause of the trouble, and proceeded to investigate the matter with characteristic thoroughness, with a view to the possible utilization of this organism as a means of controlling the chinch bug in the field. After a careful study, however, he was finally forced to the conclusion that the bacteria were not parasitic upon the chinch bug at all, but that they were really normal to the cæcal appendages of healthy bugs and that they probably had some important function in the metabolism of the insect. He also established later, by the examination of a great variety of insects of different orders, and especially of Heteroptera, that the chinch bug was not unique in this regard, but that the same phenomenon also occurred in a number of other species of Lygæidæ as well as in representatives of several other families of Heteroptera, and that wherever the cæca were present in this group, they were always filled with specific bacteria. A large part of this work was never published and the unpublished notes, which proved to be of the greatest assistance in this work, were turned over to me by Professor Forbes for use in continuing a study of the subject.

The problem as it was suggested to me by Professor Forbes was primarily to determine the significance of the presence of these "normal" bacteria of the cæca—to work out, if possible, the relation existing between the insects and their characteristic intestinal flora, and also, indirectly, to determine whether the occurrence of these complex cæcal structures might not throw some light on the phylogeny of the Heteroptera.

The following study was carried on under the supervision of Professor Forbes, and I consider it an honor to be in a position to express my indebtedness to him for his constant aid and encouragement throughout the course of the work.

I am also greatly indebted to Dr. W. J. MacNeal, in whose laboratory I was very kindly permitted to do a considerable part of the work on the bacteriology of the subject, although at that time the laboratory was so crowded that few men would have thought of admitting me.

Constancy of the Cæcal Infection in Heteroptera.

One of the first questions that came up in connection with the work was that of the constancy of the bacterial infection of the cæca—whether these organs were always infected and whether the infecting organism was always the same. It had been established by Professor Forbes that the infection in *Blissus leucopterus* was constant throughout the range of this insect in Illinois, and all the Heteroptera in the neighborhood of Urbana, in which the cæca were present, also showed the infection; but to make certain that this relation was one of fundamental significance and that the infection was not merely a local phenomenon, peculiar to the Heteroptera of Urbana, or perhaps of Illinois, it was planned to select some common, widely distributed species and to examine specimens from as great a range as possible.

It was necessary, of course, that the species selected for a study of this kind should be widely distributed as well as common and easy to collect and, what was of even greater importance, that the cæcal bacteria should be characteristic in form, to insure that the infection was not due to different species of bacteria of similar form occurring in different parts of the range of the insect.

The harlequin cabbage bug, *Murgantia histrionica*, was finally chosen as the species most nearly fulfilling these conditions. It ranges from California across the southern half of the United States and thence to New England; and it is usually a very common and conspicuous form wherever cabbage is grown; but what made it especially suitable for this test was the large size and remarkable form of its cæcal bacteria (Plate VIII., Fig. 26). Instead of being minute, short rods, as in *Anasa tristis* and *Blissus leucopterus*, the only two other species available for the test, those of *Murgantia* are very large, contorted, spirochæte-like forms which could not possibly be mistaken for any of the other varieties of cæcal bacteria.

To secure specimens, requests for living material were sent to a number of station entomologists located in parts of the country from which *Murgantia* had been reported. Living specimens were thus secured from ten states, extending in a continuous series along the southern and eastern part of the United States for a distance of between two and three thousand miles, including California, New Mexico, Oklahoma, Arkansas, Mississippi, Alabama, Georgia, South Carolina, North Carolina, and Maryland. The specimens of *Murgantia* for this study were obtained through the kindness of the following entomologists—the locations given showing the place where the insects were collected:

T. B. Symons, College Park, Maryland.
Franklin Sherman, Jr., Greenboro and Raleigh, North Carolina.
A. F. Conradi, Clemson College, South Carolina.
H. P. Stuckey, Experiment, Georgia.
W. E. Hinds, Auburn, Alabama.
R. H. Harned, Agricultural College, Mississippi.
Paul Hayhurst, Lanske, Arkansas.
C. E. Sanborn, Stillwater, Oklahoma.
Fabian Garcia, Agricultural College, New Mexico.
C. W. Woodworth, Live Oak, California.

In addition to these localities, specimens of *Murgantia* were also examined from different parts of Illinois, especially from the southern part of the state, where they were collected by Mr. L. M. Smith, of the state entomologist's office; and two specimens were also taken as far north as Urbana, Illinois.

Upon a comparison of the contents of the cæca of specimens from these widely separated localities, it soon became evident that the peculiar organism, first observed in the cæca of the two specimens collected at Urbana, was a constant inhabitant of the cæca of *Murgantia*, and that whether the individual examined was from California or Maryland this was invariably the highly characteristic, contorted organism first observed in the Urbana specimens.

While the cæcal organisms from all these localities are clearly of the same species, all having precisely the same typical form, there are certain occasional differences in size that it may be well to mention. It was observed, for example, that in the insects from Georgia, as well as in occasional specimens from

Illinois, the bacteria, although characteristic in shape, often averaged decidedly longer and stouter than those from most of the other localities; but this difference in size was not constant in all the specimens of *Murgantia* from these states, and it was doubtless due to some slight difference in the metabolism of the particular individuals examined.

Murgantia is the only species that I have studied in any numbers with this idea in mind, from outside Illinois; but hundreds of specimens of other species have been examined from different parts of this state, and all of these show, without exception, that in their morphology the type of cæcal organism is absolutely constant for a given host species.

Origin of the Cæcal Infection in the Heteroptera.

At an early stage in the work it became evident that one of the first things to be determined was the exact time and manner in which the infection with the cæcal bacteria normally takes place. Assuming that the normal bacteria would develop readily in artificial cultures, the next essential step would be to differentiate between this organism and any of the common saprophytic or parasitic forms that are so frequently met with in the alimentary canal of most insects, and which would naturally be expected to appear also in cultures from the cæca. Until the identity of the normal species in culture could be established beyond question, it would clearly be useless to undertake any time-consuming study of the physiology of any of the organisms that might be isolated in an attempt to correlate this with the digestive processes in the insect; and at the commencement of the work it appeared that the only possibility of certainly differentiating between the normal bacteria and the contaminating forms that might appear, was in some way to secure insects free from the infection, and then by direct feeding experiments to determine which were really the "cæcal" bacteria.

The normal bacteria are present, not only in the cæca, but also in considerable numbers free in the alimentary canal of the host and, as might be expected, they also occur in the brownish, liquid excrement of the insect. It is easy to imagine from this how the surroundings might be very highly contaminated with this organism, especially in the case of many of the common gregarious, phytophagous Heteroptera, such as the chinch bug, the squash bug, or *Murgantia*. It is a very common habit with these insects, as I have frequently observed, especially when in the immature condition, to sample with their beaks any drop of liquid that they may find; such as drops of dew, drops of sap exuding from wounds in the host plant, or even the liquid excrement from other individuals.

From these facts, and since the bacteria appeared to be limited to the alimentary canal of the host, it was assumed that the infection probably occurred by way of the mouth and that the alimentary canal was doubtless invaded at some time during the early nymphal life of the insect.

Proceeding on this assumption, it was planned to rear the insects from the egg in sterile cages in order to have material for use in infection experiments with the different organisms that might be isolated from the cæca. *Anasa tristis* was selected at first for this rearing work, since the eggs of this species are very easily obtained in quantities at any time during the summer, and also because they are perfectly smooth and readily sterilized. As it was planned at first, the rearing of these sterile bugs should have been a comparatively easy matter. Since it was found that this insect is able to live and develop fully as well on the squash itself as upon the sap of the vine and leaves, the only essential equipment for a breeding cage would have been very simple indeed. The breeding cage, as I had intended to arrange it, was to have been merely a rather wide-necked Ehrlenmeyer flask of something like 500 c.c. capacity, plugged with cotton and sterilized. Small pieces of squash, removed aseptically, were then to be introduced into the flask, together with the sterilized eggs of the squash bug.

As a preliminary to this breeding work, experiments were carried out to determine whether aseptic material could be readily secured from the squash, and especially whether the eggs of the squash bug could be sterilized effectively without interfering with the subsequent development of the embryo. It was found that with a little care, pieces could be removed from a squash without any risk of contamination; and it was also found that the development of the eggs of the squash bug was in no way hindered by a very thorough treatment with a solution of mercuric chloride. When such eggs were placed in bouillon as a check on the thoroughness of the disinfection, they would remain apparently sterile for a period of three days or more and then, at about the time the experiment should have been considered a success, an abundant growth would suddenly appear which apparently consisted of a number of different contaminating organisms. This test was repeated a number of times, with certain variations in the method of disinfection. It was thought at first that perhaps the film of air surrounding the egg prevented the aqueous bichloride solution from doing effective work, and the eggs were accordingly first moistened with alcohol to remove the film of air; but no matter how thorough the disinfection was made, the confusing growth almost always appeared.

Since there could be no question but that the surface of the eggs had been completely sterilized, there remained nothing but to conclude that the contaminating organism or organisms were within the egg itself.

As is well known, the eggs of birds may sometimes contain bacteria while still in the oviduct, and before the shell has been laid down, so that when the shell is formed they are included within it. There is no reason why the egg of an insect should not become contaminated in much the same way, and it was thought at first that this must be the case with the eggs of the squash bug. This species was accordingly abandoned, since it was clearly out of the question to rear sterile individuals of it. A little later in the season a few eggs were secured from specimens of *Murgantia histrionica* that had been received from Dr. W. E. Hinds, of Auburn, Alabama; and it was decided, as a last resort, to test these to see if they were also contaminated. These eggs were given exactly the same treatment as had been given those of the squash bug; but instead of developing the contamination that appeared so constantly in the squash bug eggs, they invariably remained sterile and showed no growth whatever, even when crushed immediately upon being placed in the bouillon.

Before undertaking the rearing work on *Murgantia* that was logically opened up by these negative results, it was decided to determine just how long after hatching the infection really occurs under normal condition. A fairly complete series of the immature stages of this insect was available at the time, and starting with a half-grown nymph, the cæca of successively younger individuals were examined until a stage only a few hours old was reached without finding a single specimen free from the infection. The next step was to examine a nymph immediately after hatching and before there was any possibility of its having fed; and as the cæca of this specimen also proved to be infected, it became evident that the cæcal organism itself must be transmitted directly through the egg.

In order to establish this point beyond question, embryos of *Murgantia* in different stages of development were examined, and it was found that in an embryo taken as early as forty-eight hours before the time for hatching, the organism which had been found uniformly in all the post-embryonic stages of the insect also appeared in the embryo. The bacteria appear in that section of the gut which is due to develop into the cæcal apparatus of the adult insect and which, owing to its pink color, can readily be distinguished from the other embryonic divisions of the alimentary canal.

While the bacteria from the embryo of *Murgantia*, twenty-four to forty-eight hours before hatching, are comparatively very few in numbers, they nevertheless show the same characteristic, spiral forms that are met with in the adult, although those from the embryo are usually much shorter and decidedly more difficult to stain. A considerable number of embryos of this insect were examined in this way and, without exception, the cæcal region in all was infected with the same peculiar contorted organism.

These observations on *Murgantia* naturally suggested that the apparent contamination from the eggs of the squash bug might also have been due to the cæcal bacillus of this insect carrying through the egg in a similar manner; although the fact that no growth was secured from the eggs of *Murgantia*, in which the cæcal bacteria had been demonstrated microscopically, was rather unfavorable to this view. This fact also seemed to indicate that perhaps the cæcal organism was so closely adjusted to conditions in the body of the host insect that it would not develop readily on artificial media.

At this time no more eggs of the squash bug were available, and the cultures from them had all been discarded as of no significance; but the following season, when the matter was again investigated in the light of these new facts, it was found that the cultures that developed so regularly from the eggs of this insect were identical with those that were also secured from the cæca, and that instead of containing a number of contaminating organisms, the growth really consisted of a pure culture of the cæcal bacillus. The reason for believing, at first, that the cultures from the eggs represented more than one organism and the fact that no growth was secured from the eggs of *Murgantia* are discussed in connection with the regular culture work; and it is enough to say here that when ordinary care was taken in the disinfection of the eggs of *Anasa tristis*, the resulting growth was invariably a pure culture of the cæcal bacillus.

It was also established for a number of other species, including the lygæid, *Blissus leucopterus*, that the bacteria are present in the cæca of the nymph immediately after hatching, and consequently, at a time when all outside contamination could be excluded; but it was considered a piece of unnecessary routine to attempt to carry the examination back into the embryo of these forms as had been done in the case of *Anasa tristis* and *Murgantia histrionica*.

The exact manner in which the cæcal association developed in these insects can, of course, only be surmised. It is possible that the present normal bacteria were formerly pathogenic for the host, and that the cæca, as they now exist, originally developed from true pathological structures formed as a result of the invasion of the tissues by these organisms; or the bacteria may originally have been merely saprophytic forms, peculiar to this section of the alimentary canal, which gradually became adjusted to an existence in the cæca.

One factor that might be regarded as supporting the first view is the fact that the bacteria are, to a certain extent, intracellular; and it is easy to imagine how these organisms, originally attacking certain of the epithelial cells of the gut, might have stimulated the formation of pathological cæca, which, being incidentally of some use to the host, were preserved and even greatly elaborated in structure. There is somewhat better reason for believing, however, that the cæca were originally independent organs which finally came to be occupied by the normal bacteria, and this subject will be taken up under the discussion of the cæca themselves.

That the infection is regularly transmitted through the egg from generation to generation is very evident from the above facts; but this does not necessarily mean that the organism is so closely bound up with the host as to exclude the possibility or even the importance of infection from without. One can hardly believe that reinfection by way of the mouth, whether an important factor in the association or not, is not constantly taking place, at least from the excrement of other individuals if not also from some undiscovered form of the organism that may exist normally as a free saprophyte.

As is well known, certain pathogenic blood parasites, as the spirochætes, are regularly transmitted through the egg from one generation to succeeding generations of the intermediate host, which is a tick in the case of *Spirochæta duttoni.* It has been established, in this particular case, not only that the mother that swallows the infected blood may transmit the parasites to her young, but that their descendants, although allowed to feed only on clean animals during their entire lifetime, may also pass this second-hand infection on to their young. Transmission may not stop even here, as it has been clearly established that it may continue in this way for at least three generations of ticks.

Just why this process should not go on indefinitely, when once the infection is acquired by the tick, is not clear; but evidently it does not, as only a comparatively small per cent. of the individuals of *Ornithodorus moubata* are able to transmit the disease to man.

In order for the association to continue, repeated infections with the blood-inhabiting forms of the spirochætes must apparently take place. It may be that conditions in the arthropod host are unfavorable in some way to the spirochætes, and that they gradually degenerate in passing through a number of generations of the host, so that for a continuance of the association a periodical reinfection with a more vigorous strain from some outside source is required. While the occurrence of a similar relation in the Heteroptera is by no means certain, there are a number of facts pointed to both by direct observation and by analogy, that would seem to favor this view rather than the theory that the bacteria are confined strictly to their insect hosts and are transmitted indefinitely from generation to generation through the egg.

One reason for the first view is that in certain of these insects, as in *Murgantia* and in many other pentatomids, the bacteria really appear to be existing under marked difficulties of some sort, as indicated by the proportion of involution forms and the constancy with which they appear in the cæca of these insects, and also by the fact that the vast majority, at least, even in such forms as the squash bug, are apparently unable to develop any longer or artificial media. In the case of this particular insect, while growth was uniformly secured from the cæca where liquid media was used, it soon became evident, when plate cultures were made direct from these organs, that but a minute fraction of the bacteria actually present were able to develop on artificial media, the vast majority evidently being either dead or modified in such a way by their existence in the cæca that they were wholly unable to develop outside the host insect. Frequently when only a very short section of the cæca was removed for inoculation, especially where plate cultures were made, no growth whatever developed, although there were certainly thousands of bacterial cells introduced.

Another case that may throw some light of analogy on this question is that of the well-known symbiotic relation between certain green turbellarians and their associated algæ (Keeble, '07). Numerous attempts have been made to cultivate these algæ on artificial media, but uniformly without success. This was sometimes explained on the theory that these two organisms had been associated in this relation for so long that the alga had completely lost its ability to develop in the free state, and was now totally dependent on the animal, as is often the case in many of the more highly specialized parasites. This is certainly a very reasonable view but it has recently been discovered that the zoöchlorellæ, although exerting a profound influence on the metabolism of the host, do not represent the typical organism any more than the *Leptus irritans* stage could be taken as representing the true life cycle of the particular species of *Trombidium* to which it belongs. The zoöchlorellæ, like *Leptus irritans*, really represent an abnormal departure from the regular development of the species. The form to which these symbiotic algæ belong is really a typical, free-living, flagellated organism, which is normally free during its entire, regular life cycle, the zoöchlorella stage representing merely those individuals which chance to be swallowed by the worm. The free-living stage of this organism will grow vigorously on artificial media and infection is found to take place readily from pure cultures, but in the case of the organism direct from the tissues of the host worm it has been found not only that they will not develop on artificial media, but that they have been so modified by their short existence in the body of the host, that they are not even able to set up the infection in clean worms although they may be swallowed in quantities.

In certain cœlenterates the relation of the alga to its host animal appears to be better established than in the planarians. In *Millepora* (Mangan, '09), at least, the algæ are found in the egg and appear to be transmitted through it for at least one generation; although there is a possibility that the inclusion of these organisms in the egg is a mere accident and that the chief source of infection is the free-living stage of the alga which is continually being swallowed by the animal.

The only other well-known bacterial infection in insects directly comparable with that in the Heteroptera is one whose relations have been worked out by Petri for the little olive fly, *Dacus oleæ*—a subject which will be discussed in detail in another place. In this insect the bacteria, which are also intestinal forms, are present in the larva as well as in the adult and, according to Petri, there is a complicated modification of the ovipositor which functions as a secondary reservoir for the intestinal bacillus. Petri at first very naturally reasoned, since these bacteria were present in larvæ that had hatched from eggs laid by sterile flies in sterile olives, that the organism must be transmitted through the egg; but in his later work he concludes that this is not the case, and he finally decided that infection normally took place in the following manner: When the fly deposits its egg in the fruit of the olive, a quantity of the bacteria from the special structures in the base of the ovipositor are also introduced and infection only takes place when the young larva, upon hatching, swallows some of the surrounding bacteria. Petri also isolated a chromogenic organism from the soil of olive orchards and from various parts of the olive tree which he regards as identical with that from the intestine of *Dacus*, and he seems to think that the insect originally developed its infection from this free, saprophytic form.

The organism isolated so uniformly from the eggs and cæca of *Anasa tristis* clearly belongs in the large group of fluorescent bacteria that are so common in water and in soil generally. A number of strains of these saprophytic fluorescent organisms have been isolated and studied from the soil about squash vines and from the squashes themselves; and they clearly belong in the same group with the cæcal bacillus of *Anasa tristis*, although certainly none of them are identical with it.

Cultivation of the Cæcal Bacteria.

In undertaking a study of the physiological relation existing between the Heteroptera and their cæcal bacteria, it was very evident that the whole question hinged on the cultivation of these organisms, and that little or nothing could be expected from such a study until pure cultures were obtained. The fact that these were really normal bacteria and occurred in every individual possessing the cæca, in itself presented a very serious obstacle to the culture work, since this very fact apparently excluded all possibility of confirming, by direct infection experiments, any cultures that might be obtained from the cæca. An attempt was made, as has already been mentioned, to rear sterile individuals from the egg in aseptic cages, in order to secure material free from the cæcal bacteria for these infection experiments; but since the bacteria were found to pass normally through the egg, these rearing experiments were unsuccessful; and this apparently left no alternative but to select for the culture work only those insects in which the bacteria were so characteristic in form that they could readily be recognized in mixed cultures.

Since all possibility of checking the cultures by infection experiments had to be definitely abandoned, there was very little inducement, at least at first, to attempt the cultivation of these bacteria from the Coreidæ or from such lygæids as *Blissus leucopterus*, in all of which they are short, uniform rods with nothing morphologically to distinguish them from dozens of other saprophytic bacteria that might occur as contamination in the cultures. While these insects appeared wholly unsuited for preliminary culture tests, many of the Pentatomidæ, as *Peribalus limbolarius*, *Brochymena quadripustulata*, and especially *Murgantia histrionica* were apparently ideal for this purpose, as the bacteria which they harbor, instead of being the small, typical bacillus form of the Coreidæ and of most of the Lygæidæ, are very characteristic in appearance, varying from the extremely long, straight, rod-like forms of *Peribalus* through the short, uniformly bent organisms of *Brochymena* to the remarkably large, characteristically contorted form uniformly occurring in *Murgantia*.

For this apparently good reason, the culture work was, at first, concentrated especially on *Murgantia histrionica* and a few other pentatomids; for it was very clear that if the cæcal bacteria from these insects would develop on artificial media at all, and still retain the characteristic form as they occur in the cæca, there would be no difficulty whatever in differentiating them from any possible contamination that might appear.

In the larger Heteroptera, as well as in those of moderate size, it was found that by careful dissection any division of the alimentary canal could be removed with practically no danger of outside contamination; the peculiar shape and structure of the abdomen in these insects being especially adapted to this operation.

For removing the cæca aseptically it was found, after a number of methods had been tested, that the following simple procedure was really the most satisfactory. The insect is first lightly chloroformed to prevent struggling, the wings are clipped off near the base and the whole body moistened with alcohol to remove the film of air and allow the penetration of the bichloride solution which was usually used in the proportion of 1–500. The mercuric chloride solution is best applied with a bit of absorbent cotton held in a pair of old forceps. In this way the entire body of the insect can be thoroughly scrubbed with the disinfectant, so that any folds, such as those between the body segments, will certainly be moistened. After the bichloride solution has completely dried, which may be very well hastened by passing the insect back and forth before a Bunsen flame, the flat edges of the abdomen are clipped off, from near the posterior end up to the thorax, with a pair of fine scissors which have been previously flamed. The top of the abdomen immediately back of the thorax may be cut across with sterile scissors and the resulting flap formed of the entire dorsal wall of the abdomen may then be lifted back with a pair of flamed forceps, leaving the abdominal viscera exposed.

Usually in forms such as the larger Coreidæ and Pentatomidæ, the alimentary canal is considerably coiled in the posterior half of the abdominal cavity, and is covered above by a thin layer of fatty tissue which must be moved to one side before the cæca can be reached.

The cæca may be readily distinguished from the other divisions of the alimentary canal, as they are pure glistening white in the Coreidæ or tinted yellow or pink in many of the Pentatomidæ.

In making cultures from the cæca, the usual procedure was to open the abdomen with sterile instruments, in the manner just described, and after removing the dorsal fat body, to clip out a small section from near the middle of the cæcal system, which was then quickly removed with flamed forceps to a tube either of sterile salt solution or bouillon, the final cultures being made from this tube.

For reasons which have already been explained, the first serious attempt at cultivating the cæcal bacteria was made with *Murgantia histrionica;* and unfortunately the work was confined for a long time to this species and a few other pentatomids, in an attempt to discover some means by which their peculiarly characteristic bacteria could be made to develop on artificial media.

When the work was first undertaken with *Murgantia,* it was planned to remove a section of the cæca to a tube of sterile salt solution or bouillon as just described, crush it, and from this make plate cultures in the usual manner, it being fully expected that a number of doubtful forms of bacteria would be isolated as contamination from the ordinary transient intestinal flora assumed to be present in the insects. It was a decided surprise, however, when not only the typical cæcal bacteria failed to appear in these plates, but no contaminating forms even developed. This was repeated with large series of the insects but the results were always the same, the cultures remaining sterile with a discouraging regularity. It was thought at first that perhaps the liberal amount of alcohol and bichloride solution used in disinfecting the outer surface of the insects might possibly have penetrated the tracheal system and that enough had been removed with the trachea adhering to the cæca to prevent all growth in the cultures, although this seemed hardly possible. Tests were made, however, in which only those parts of the insect which were actually to be cut were moistened with the disinfectant, the spiracles being avoided entirely, and the results were exactly the same, the cultures remaining uniformly sterile. It was later found also that without the use of any disinfecting solution the glands could be removed with no fear whatever of contamination from without, provided the scissors with which the cutting was to be done were used hot enough to sear as the body wall was opened; the cuts being made as rapidly as possible to avoid the danger of heating the tissues too deeply.

While the apparent incapacity of the cæcal bacteria from this insect to develop on ordinary media was somewhat discouraging, at least one very important fact was brought out by it. Since no growth at all developed from the cæca on ordinary media, it was very clear that, contrary to what had been expected, there would be no complication of contaminating organisms from the intestine which are so common in many other insects, since in *Murgantia* these forms seemed, for some very definite reason, to be wholly absent; and it followed that, if by any means conditions could be made suitable for the development of the cæcal bacteria of this insect on artificial media, the difficulty of proving that the organism in culture was really the one sought and not some accidental contaminating form would be wholly avoided.

With this as an encouragement, an excessive amount of time and effort was wasted in an attempt so to modify culture conditions that the normal cæcal organism of this insect could be induced to grow on artificial media. Since, in reaction, the cæca appear faintly alkaline, the different media used were usually made neutral or slightly alkaline; and as the normal food of the insect is cabbage, this was largely used in the different media tested; but as all the results were uniformly negative, no minute discussion will be given here of the different modifications that were tried. Anaerobic cultures were also made with negative results, although at the time, this test seemed almost superfluous in view of the abundant tracheation of the cæca.

After the failure of these direct culture experiments, it was reasoned that upon the death of the insect the bacteria probably became gradually adapted to a saprophytic mode of life, and that by taking advantage of this they might still be forced to grow on artificial media.

In testing this hypothesis, several series of from forty to fifty insects each were used. The insects were killed either with chloroform or by grasping the head for an instant with a pair of very hot forceps, the last method being the one most generally used and proving a very convenient way of killing the insect without breaking the body wall. The dead insects were then thoroughly sterilized by washing in a mercuric chloride solution after the removal of the wings, and after drying they were placed in small, tightly stoppered, sterile vials, to prevent drying of the internal structures, and kept for three or four days in a cold box at about 20° C. At the end of this time the alimentary canal was usually intact and showed no invasion by foreign bacteria, while the cæcal bacteria themselves showed no perceptible changes either in numbers or in the invasion of other regions. Bouillon tubes inoculated from the cæca of these dead bugs usually remained sterile, but growth appeared in two or three per cent. of the tubes, and it was thought at first that this growth might represent strains of the cæcal organism which had been modified by their stay in the dead bugs so as to develop on artificial media. When these cultures were examined under the microscope, however, they showed, not the long, irregularly bent organism from the cæca of *Murgantia*, but what appeared to be a pure culture of a very short, actively motile, fluorescent bacillus, which grew vigorously on ordinary media. Although this organism was obtained in culture from ten or more of the dead insects and appeared to be the only one that developed, it was so different from anything that had been expected from the cæca that it was regarded as one of the common fluorescent water bacteria that had probably been present in the anterior part of the alimentary canal and which, after the death of the insect, had invaded the cæca. The culture work on *Murgantia* was discontinued here, as it apparently promised nothing to warrant any further work.

In the meantime repeated attempts were also made to cultivate the cæcal bacteria from a number of other pentatomids, including chiefly *Peribalus limbolarius*, *Cœnus delius*, *Brochymena quadripustulata*, *Euschistus variolarius* and *Mormidea lugens*, but the results, as with *Murgantia*, were uniformly negative, neither the cæcal organism nor any contamination appearing in the cultures from any of these species, except in exceptional cases where the technique was clearly at fault.

Common forms such as the squash bug and the chinch bug had been purposely avoided in this work owing to the obvious impossibility of distinguishing between ordinary contaminating organisms and the cæcal bacteria typical for these insects; but since the negative results from the work with *Murgantia* and the other pentatomids showed clearly that the cæca, in these insects at least, were wholly free from foreign bacteria, the possibility of using such insects as *Anasa* in culture work did not seem so hopeless as at first, especially if it was found that this statement applied also to them.

As a last resort it was decided to attempt the cultivation of the cæcal bacteria from *Anasa tristis*, this species being selected because it is fairly large and is usually abundant and readily obtainable in winter as well as in summer.

In the preliminary work on this species, pieces of the cæca were removed and dropped at once into tubes of squash juice bouillon, the composition of which was the same as that of ordinary beef juice bouillon with the addition of a decoction from 150 grams of squash stems and leaves per liter.

In the squash bug the cæcal bacillus is a very short, uniform rod, averaging 0.9 micron long by 0.7 micron wide. As they occur in the cæca these bacteria do not show the slightest indication of motility; and they are usually arranged in pairs, or they may, exceptionally, form short chains of from three to four or more individuals.

In undertaking the culture work with this insect I had very little hope of growing the cæcal bacteria successfully, and really did not expect more than a repetition of the negative results secured with the Pentatomidæ, but I nevertheless thought that even the relation shown there would be worth establishing in other Heteroptera.

The first culture experiments with *Anasa tristis* were undertaken with seven adults that had been kept in a warm room for nearly a month. Of the tubes inoculated from the cæca of these seven insects, every one developed growth; and in each case this appeared to be a pure culture of an organism morphologically very similar to the bacteria in the cæca; but as these insects had been feeding on partly decomposed squash a short time before the dissections were made, it was realized that the growth might very easily have been due to some foreign organism swallowed by the insects while feeding. Nevertheless, the fact that the growth in all the tubes was apparently the same, was very encouraging in view of the uniform failure to secure any growth whatever from the Pentatomidæ.

To ascertain whether or not this growth was merely a contamination from the squash, or whether the cæcal organism could still develop saprophytically, as the cultures suggested, a series of fifty specimens of *Anasa* were taken, fed on perfectly fresh squash for two weeks and then kept for a full week without food, in order to give the cæcal bacteria sufficient time to destroy any contaminating organisms that might have been swallowed while feeding.

Cultures were made from this series and, to my surprise, growth developed in all fifty of the tubes and appeared in each case to be a pure culture of a short, motile organism identical in size and form with the bacteria in the cæca of this insect, and so far as could be determined these were the same as the organism that had appeared in the cultures from the first seven insects tested.

This series of experiments appeared to show very conclusively that we had at last succeeded in growing the cæcal bacteria in pure culture, provided of course that foreign bacteria were excluded as completely from the alimentary canal of *Anasa* as they had been shown to be in the Pentatomidæ. Subcultures were not started from these tubes for seven or eight days and upon examining them again at the end of this time, a number were found to contain organisms so different from the cæcal bacteria that there was apparently no escape from the conclusion that the cultures had become in some way contaminated. When examined upon the first appearance of growth, nothing was found but the short uniform bacillus, which was as much like the bacteria direct from the cæca as could be imagined; but at a later examination two apparently distinct contaminating forms were discovered. One of these apparent invaders was a fairly uniform rod-shaped bacillus 4–8 microns long by 1 micron wide, so much larger and longer than the typical cæcal organism that the two could be distinguished at a glance. The other was a perfectly spherical form, 0.5–0.7 of a micron in diameter, which occurred commonly in extremely long chains of a hundred or more individuals.

Contamination from some source had apparently taken place in a number of tubes, and it was therefore decided to discard the whole series rather than attempt to make a detailed study of the different forms that might be isolated from the cultures in an attempt to connect some one of these with the species from the glands—an undertaking evidently little short of hopeless. Another cause of uncertainty here was the fact that the cæcal bacteria from the Pentatomidæ would not develop on ordinary media; and as there was no reason whatever for assuming that the forms from the Coreidæ would behave any differently, there was clearly a possibility that the growth that had appeared in all the tubes was really contamination and that the true cæcal bacillus was not represented here at all.

If the property of totally excluding all other bacteria from the alimentary canal did not hold for the cæcal bacteria of the

Coreidæ, as it very clearly did for those of the Pentatomidæ, then there was certainly very little encouragement to continue culture work with these insects, for although strains of bacteria might be isolated that would agree perfectly with the cæcal bacteria in their morphological characters, this would establish very little, as there is nothing about the bacteria from the Coreidæ to distinguish them in this way from many common saprophytic forms that might readily gain access to the intestine of these insects.

As the first two sets of culture tests were made from bugs that had been kept in a warm room and had been feeding continually up to a week of the time before the dissections were made, it seemed possible that the antagonism of the coreid bacteria to invading forms was not so absolute as in the Pentatomidæ, and that, if hibernating insects were used which had had no opportunity to feed for a considerable period, the normal bacteria might in this time have succeeded in effectually killing off the foreign species. It was accordingly decided to attempt the cultivation of the squash bug bacillus once more, hibernating insects being used this time in the hope that as a result of long fasting their normal bacteria might have eliminated the transient forms.

For these tests, the insects were taken after the middle of December from their winter quarters. They could not have fed for at least a month; and they were dissected at once. Thirty-five specimens of *Anasa tristis* were collected for this work and every precaution was taken in the dissections and inoculations to guard against contamination from without. The insects were sterilized before dissection with a thoroughness which would not have been permissible with *Murgantia;* but, notwithstanding this, cultures were obtained from these insects as regularly as they had failed where *Murgantia* was used, all thirty-five of the tubes inoculated developing an abundant growth.

These cultures were incubated at room temperature and were watched closely for the appearance of any contamination. Growth was found to appear rather tardily, developing in from two to four days, and at first, as in the two previous series of experiments, consisting of an apparently pure culture of a short, motile organism which could not be distinguished morphologically from the cæcal bacteria as taken direct from the insect. In a few days after the first appearance of growth, however, forms were observed in many of the tubes which were clearly not of this type. In about a week the two abnormal forms which had appeared in the preceding experiment were observed in a number of these tubes; and, in addition to these, a third type was also discovered which appeared in several of the tubes as a very large, oval, yeast-like organism often over 4 microns long by 2–3 microns wide.

As all contamination from without had been excluded beyond question, all of these unusual forms must certainly have come from the cæca; and as nothing resembling any of them had ever been observed in these organs by direct examination, their appearance in the cultures was very difficult to explain.

Upon close examination it was seen that these contaminating organisms were not strictly constant in form, although it did not seem possible at first that these strikingly different organisms could be involution forms of the bacillus that first appeared in cultures from the cæca. The yeast-like bodies were usually free, with one or more small typical buds at the ends; but occasionally one or more of these large bodies could be seen in the long chains of small coccus-like forms; and the large bacillus was not always a typical rod, but very often tapered at the ends very decidedly, and even merged into bodies that resembled the yeast-like forms. Since it seemed possible that some of the contaminating organisms were merely involution forms, and not independent organisms, as had been assumed at first, it seemed worth while to make a thorough study of the different forms occurring in the cultures to determine how much normal contamination there really was in the alimentary canal of these insects. Plate cultures were accordingly made from some of the tubes that appeared most highly contaminated, and these were searched very carefully for different kinds of colonies; but so far as could be determined by direct examination, the colonies were invariably all alike.

Cultures were made from large numbers of these colonies in an attempt to isolate the different contaminating organisms that had appeared in the original bouillon tubes. All the subcultures from the plates invariably developed into the short form resembling the cæcal bacillus; but when subcultures were made from these into bouillon, all the abnormal forms regularly appeared, as they had in the cultures direct from the cæca. Since each colony in the plates contained only descendants of a single bacterial cell, it became very evident that the apparent contamination from the cæca of the squash bug was nothing more than an extreme case of the production of involution forms, and that but a single organism had ever really developed in cultures from the cæca of these insects. This established conclusively the fact that we had but one organism to consider in the culture from the cæca of *Anasa tristis* although it did not show beyond question that this one organism was really the true cæcal bacillus. In view of the results from the culture experiments with *Murgantia*, however, it certainly seemed probable that this was the cæcal bacillus.

One fact which made this view rather uncertain for a time was that in 1895 a generally distributed disease of the squash bug was discovered by Duggar in the neighborhood of Urbana. This disease appeared to be fairly common, and was described as affecting principally the fat body and the perivisceral tissues generally. There was a distinct possibility that a chronic form of this disease might be present among the apparently healthy bugs that had been used in the culture tests just described, and that the growth in these cultures was really due to this organism from the fat body contaminating the cæca as they were removed from the insect for inoculation.

No more insects were available for testing this theory, but the following season several large series of culture tests were carried out with this point in view, two tubes being inoculated from each insect in the following manner: Upon opening the abdomen a large lobe of the fat body adjacent to the cæca was removed, before the alimentary canal was broken, and placed in one of two tubes of bouillon. A section of the cæcal system was then removed and put into the check tube. Without discussion of detail, it is enough to say that as a result of several hundred such tests growth was obtained from the cæca in every case, while the tubes inoculated from the fat body invariably remained sterile.

In order to ascertain the distribution of the cæcal bacteria throughout the alimentary canal, cultures were made from different sections of the midgut, including the first stomach, third stomach and the cæcal region. From a large number of such tests it resulted that while growth invariably appeared in the tubes from the cæca, and usually also from the third stomach, only from ten to twenty per cent. of the inoculations from the first stomach showed any growth, all the others remaining sterile.

Cultures were repeatedly made from the eggs of *Anasa tristis;* and upon comparison, it was found that the organism from this source was certainly the same as that isolated from the cæca, and it was also determined that this was the only organism that ever appeared in cultures from the eggs of this insect.

It was expected, in the beginning, that the bacteria from the cæca of the Heteroptera, if they developed at all on artificial media, would prove to be a number of very different forms, perhaps occurring only in their respective insect hosts, and when it was found that the organism isolated from the cæca of *Anasa* belonged to so common a group as the non-liquefying fluorescent bacteria, it was feared that, after all, the form isolated from this insect might have been merely the result of a contamination of the alimentary canal by some of the ever-present species of this group; although the fact that this same organism was isolated regularly from the egg seemed very conclusive.

In order to show beyond question whether or not the bacteria so regularly isolated from the squash bug were really the same as those normally present in the cæca, it was planned to check the cultures which had been obtained from the insects against the bacteria direct from the cæca by means of the agglutination test. Young rats were chosen as best suited for this work, as they are much more hardy than young guinea pigs of the same weight. The animals selected weighed only from fifty to seventy-five grams, as it was feared that sufficient material for the immunization might not be available in case large animals, such as full-grown guinea pigs, were used. It was planned at first to immunize the animals to cultures of the bacillus isolated from the cæca, and to test such sera against emulsions of the bacteria direct from these organs, but owing to the fact that bacteria freshly isolated from tissues do not usually react so readily to the agglutination test, the process was finally reversed and the animals were immunized with material direct from the cæca, the crushed cæca really representing a greater bulk of bacterial cells than of insect tissue.

For this purpose the cæca were removed from hibernating insects and crushed very thoroughly by rolling between sterile slides. The bacteria thus liberated were then washed off the slides and collected in sterile vials which were at once immersed in a water-bath and kept at 54° C. for thirty minutes to kill the organisms, it having been found that this temperature was sufficient for the purpose.

Material prepared in this way was injected intraperitoneally, each animal receiving five graded doses at intervals of a week or ten days, the doses varying from the cæca substance of ten bugs for the first injection to that from thirty insects for the last.

The immunized animals were killed about ten days after the last injection, the blood was drawn aseptically from the heart, and the serum stored in capillaries having a capacity of two or three drops each. Since the normal serum of many animals is known to agglutinate certain strains of bacteria in low dilution, I did not know what to expect in a case like this, and to guard against any possible error, a normal rat was bled whenever an immunized animal was killed and the sera from the two were checked against one another in every test made.

A large series of cultures from the cæca of *Anasa tristis* were tested out against such immune sera, and from the very first of these tests it was evident that the organism in culture was undoubtedly the identical form in the cæca, for they were agglutinated readily by the immune serum of the cæcal bacteria in dilutions as high as 1–500. No dilutions higher than this were tried, but from the readiness with which clumping was produced in this concentration, we may infer that the reactions would certainly have been reliable in dilutions twice as great.

In this work the macroscopic method was relied upon almost entirely, the dilutions being made in small, 6 or 7 mm., test tubes. It was found that while serum from the immunized rat would regularly produce complete clumping in high dilutions in three or four hours, when incubated at 35° C., the normal serum in these same dilutions gave no trace of the reaction. It showed no more flocculation than regularly took place in the untreated checks, although there does often appear to be a slight reaction in dilutions as high as 1-20 where normal rat serum is used.

From a careful study of the bacteria as they occur in the cæca of the different Heteroptera, supplemented by the results of this culture work, it seems highly probable that many, and perhaps all, of the different and highly characteristic forms observed in the cæca of so many of these insects are really abnormal involution forms of an organism presumably something like the cæcal bacteria of *Anasa tristis*. This cannot be certainly determined, however, until at least a few of these peculiarly shaped organisms have been cultivated beyond question. It will be remembered that during the culture work with the cæcal bacteria of *Murgantia histrionica*, growth was secured from a small per cent. of the insects that had been dead for some time. The organism isolated in this case being a short, motile form, very similar in the morphological and cultural characters recorded to the bacteria from *Anasa tristis*. As there appeared at that time, however, no way of determining positively whether these really represented the true cæcal organism of the cabbage bug or were merely accidental invaders which had appeared after the death of the insect, the cultures were all discarded, as has already been mentioned, after having been studied in only a very superficial way.

Evidently the only method available in a case such as that described for *Murgantia* is the agglutination test as applied to the cultures from *Anasa tristis*—immunization with the bacteria of the particular insect studied, direct from the cæca, and testing the resulting serum against any doubtful cultures obtained from these organs.

I have had no opportunity as yet to repeat this work with *Murgantia*, but expect eventually to make a careful comparative study of the cæcal organisms from a number of host species, concentrating especially on such forms as *Murgantia*, *Peribalus limbolarius*, *Peliopelta abbreviata*, or others in which the normal organism is equally characteristic; the agglutination test, of course, being used as a basis for the work.

It was firmly expected, when the present investigation was undertaken, that the characteristic and remarkably varied types of cæcal organisms observed in the different Heteroptera would be found to represent a large number of different species and perhaps genera, and that a very careful study and description of each type, at least as they occur in the cæca, would be necessary. In view of the results which have developed from the culture work, however, it seems probable, almost certain in fact, that all of these strikingly different forms really belong to a single clearly defined group of bacteria, and that the differences in structure that are so constant for the given host species are due to some specific physiological peculiarity of the insect which exerts a very definite influence in determining the morphology of the bacterial cell.

While all of the different strains isolated from the cæca and eggs of *Anasa tristis* belong to the group already mentioned, they are by no means all identical in their behavior on artificial media. The most striking of the differences observed was in the vigor with which they developed in culture, some strains showing a remarkably strong growth from the very beginning, others making a comparatively very weak growth even after repeated transfers, while occasionally a strain would be found which, growing very feebly at first, gradually weakened for some unknown reason and finally refused to grow altogether after three or four transfers.

Another thing that I have not yet been able to do is to make a detailed comparison of the cultural characters of the different strains isolated from the squash bug, but from the observations already made there is undoubtedly considerable variation in the minor cultural characters in many of these.

Quite recently in working with other coreids than *Anasa tristis*, cultures have been secured regularly from the cæca of *Alydus quinquespinosus*, *Aldyus conspersus*, and *Metapodius terminalis*, as well as from the eggs of the first species of *Alydus*, although cultures attempted from the eggs of a single female of *Archimerus indecorus* were uniformly negative.

While the bacteria isolated from these three coreids show certain slight differences in culture from the typical strains from the squash bug, they certainly all belong to the same group and the differences noted were no greater than those observed in different strains from the squash bug itself.

It was thought at one time the bacteria isolated from the cæca of these insects might readily be identified as some already described species known to occur free in nature, but the classification of this group was found to be in such a chaotic state that this idea was abandoned; and for the same very good reason it was thought that little would be gained by an attempt to describe them as new.

Some idea of the striking morphological differences in the bacteria from different host species can be gained from the following brief notes on a few of the more characteristic types as they occur in the cæca. No attempt will be made in this article to mention all of the many different morphological types of bacteria that have been observed and studied in the different host species dissected, the present object being merely to give examples of a few of the extreme cases that are met with. For this purpose the cæcal organisms from *Anasa tristis*, *Blissus leucopterus*, *Euschistus servus*, *Peribalus limbolarius*, and *Murgantia histrionica* should serve very well, as in this series we get the two extreme types in *Anasa* and *Murgantia*, the others representing intermediate forms.

In the first two host species the normal bacteria are much alike in appearance, both being very short rods which average something like one micron by 0.7, those from *Anasa tristis*, however, being usually slightly smaller and rather more slender than those from the chinch bug (Plate VIII., Figs. 21 and 23). In both of these insects the bacteria are quite uniform in appearance, varying no more in size and form from the typical cells than might well be expected. They occur very regularly in short chains of two, occasionally of four and very rarely of six or more cells, and do not show the slightest indication of motility as they come from the cæca. They take the common stains readily and on the whole have the appearance of ordinary bacteria with nothing especially striking to characterize them.

From *Euschistus servus* (Plate VIII., Fig. 24) the bacteria are decidedly longer than in the insects just mentioned, although they still have the appearance of ordinary bacteria. As in the two preceding forms they occur typically in pairs and show no motility. The individuals of these pairs average about 4 by 0.9 microns, while some may be considerably longer. Individual rods, which are often slightly bent, may be as long as 8 microns while short rods no longer than 1.5 microns are frequently present, although these different forms all grade insensibly into one another.

In *Peribalus limbolarius* (Plate VIII., Fig. 25) the bacteria are very much longer than in *Euschistus servus*, and while they still tend to appear in pairs this feature is not so marked as in the three cases mentioned above. The individual cells are remarkably long, varying from 5 to 50 microns long by about 1.2 microns in diameter. The shorter rods often show a characteristic bending, the curve being very gradual and extending the full length of the cell. This tendency is greatly exaggerated in the longer elements, which are frequently bent several times, but even here the curves are gradual and symmetrical, there being no sharp twisting and distortion of the rods as in *Murgantia histrionica*, and even in the longest, the diameter remains very constant throughout. The extremely long elements are more common in some individuals than in others, frequently making up a large part of the cæcal contents in such insects.

When these organisms were first encountered I could hardly believe that the extremely long, curved, thread-like structures really represented single cells and not chains of closely packed units, although no definite divisions could be made out either in fresh or in stained preparations. When stained heavily and then decolorized to a certain point these threads break up into irregular bead-like granules which are scattered rather regularly throughout. These granules clearly do not represent separate units, however, as the same bodies likewise appear in the short rods under the same manipulation, and these unquestionably represent single cells as do also the longer thread-like elements.

The cæcal organism of *Murgantia histrionica* (Plate VIII., Fig. 26) is of such bizarre form that there is really little about it to suggest its bacterial nature, and this might very well be questioned, in the absence of anything definite in the way of culture tests, if it were not that there is a more or less complete transition from it to the typical bacterial forms of such insects as *Anasa tristis* and *Euschistus servus*. The transition forms are found in such pentatomids as *Brochymena quadripustulata*, *Proxys punctulatus* and *Peribalus limbolarius*.

There is little to characterize the cæcal organism of *Murgantia* except its irregularly twisted and remarkably contorted form, which is retained not only in the cæca, but also in the embryo during its passage through the egg. The irregular form of this organism as it occurs in the cæca, which is much more suggestive of spirochætes than of bacteria, does not lend itself well to description and the reader will perhaps get a better idea of this by glancing at the figure referred to above.

These organisms vary from small, contorted individuals, 2 or 3 microns long to huge, irregularly bent bodies, not infrequently measuring 100 microns or even more in length by 1.5 to 3 microns in diameter. The most common form is 10 to 15 microns long although examples 25 to 30 microns long are common. The diameter of a single individual may vary considerably at different points and frequently such elements with a very decided bulb-like enlargement at one end, or even in the middle, occur. Others which are decidedly swollen throughout and which stain with great difficulty are fairly common, while often an end of one of the long individuals will take the stain readily, the remainder staining weakly or not at all. On the whole these organisms from *Murgantia* strongly suggest degenerating, involution forms, and this is probably their true nature.

Function and Phylogenetic Significance of the Cæca of the Heteroptera.

The midintestine of the Heteroptera is typically divided into four rather clearly defined regions which, for convenience, may be termed the first, second, third and fourth stomachs. All four can usually be made out, although in some of the more highly specialized groups certain of these divisions, especially the two posterior ones, may be very greatly reduced.

At its anterior end the midgut is uniformly dilated, forming a capacious, thin-walled, bag-like structure, capable of considerable distension. In the adult this anterior division is almost always empty, although in the nymph it is often filled, and even considerably distended, with a greenish or brownish granular mass, and, as might be expected, it is variable in size and shape in different individuals of the same species. At its posterior end this first stomach narrows suddenly and passes into the comparatively slender, tubular second stomach which usually is of uniform diameter throughout, and which empties into a second, oval or rounded, dilated portion. This third stomach, in turn, passes abruptly into the fourth and last division, which is the one that concerns us most, as it is on this section of the gut that the cæcal appendages with their normal bacteria appear.

The first stomach is almost always distinct, while the second and third may occasionally grade into each other, in the more specialized forms, in such a way that the exact line separating the two cannot be clearly made out. This fusion is brought about either by the excessive enlargement of the tubular second stomach or by the contraction of the third, or sometimes by both, and is met with only in those groups in which the cæcal appendages are wholly wanting. In those groups in which the cæcal appendages are regularly absent, the fourth stomach is uniformly reduced to an extremely short tubular portion, or it may occasionally be absent altogether, in which case the Malpighian tubes are inserted immediately below the third stomach.

In certain of the strictly predaceous groups, as in the Reduviidæ, Phymatidæ, and Acanthiidæ, the midgut is very greatly reduced in complexity, there being no trace of the fourth and very little to indicate the second and third divisions, and even the first stomach may sometimes merge into the remainder of the midgut to form an irregular tube of large caliber extending from the œsophagus to the rectum, but without any clear-cut divisions. Even in cases such as this, however, there is usually a slight enlargement at the posterior end, just in front of the point of insertion of the Malpighian tubes; and this may represent the third stomach as it occurs in typical forms.

The fourth division of the midgut is never well developed except in those families of the Cimicoidea which are provided with cæca, and so far as is known these include only the Pentatomidæ, Thyrecoridæ, Pyrrhocoridæ, Lygæidæ, and the Coreidæ. In these families, whenever the cæca are present, the fourth stomach occupies a prominent place in the midgut in the form of a long, slender tube, and it is on the posterior end of this tube that the pouch-like cæca are borne. In these forms the tubular part of the fourth stomach is regularly somewhat dilated immediately below the third stomach, and this tendency is occasionally exaggerated to such an extent that in certain species, as in *Blissus leucopterus*, a bulb-like structure may be formed at the anterior end of the tube nearly as large as the third stomach itself, but this dilation is not constant in size and does not represent one of the typical divisions of the midintestine.

The ileum in the Cimicoideæ presents a characteristic modification which may well be mentioned here, for instead of continuing as a simple tube from the point of insertion of the Malpighian vessels to the rectum, it forms a thin-walled, bladder-like reservoir, of various shapes, into which the four Malpighian tubes empty, each tube usually being inserted singly, about half way down on the side of this bladder-like ileum. The ileum opens by way of a narrow, valve-like constriction into the capacious, thin-walled, muscular rectum, which may or may not be provided with a large anteriorly projecting cul-de-sac, depending on whether the ileum opens directly into its anterior end or further down on its side as it does in some of the Coreidæ. The walls of the rectum are very elastic and its size varies greatly. When not gorged it may be no larger than the third stomach and still be smoothly rounded, while in the same species it is occasionally found so greatly distended that it occupies most of the abdominal cavity, although this condition is decidedly exceptional.

As has already been stated, the cæcal appendages of the Heteroptera are apparently confined strictly to certain families of the Cimicoideæ; and while these organs at first appear to form a number of widely different types, they are found upon closer examination to fall regularly into two clearly defined groups ranging from very simple to highly complex forms. In one of these groups in which the Pyrrhocoridæ, Thyrecoridæ, Lygæidæ, and Coreidæ may be included, the cæca are arranged in two rows extending along opposite sides of the tubular fourth stomach, in varying degrees of complexity, from an extremely simple type found in the Pyrrhocorinæ (Plate IV., Fig. 10) (in which the cæcal equipment consists merely of a double row of a half dozen or more comparatively minute outpocketings at the extreme posterior end of the tube, and this only in the female), to the highly complex arrangement met with in the Largiinæ (Plate VI., Fig. 16) and in many of the Coreidæ and Lygæidæ (Plate I., Fig. 1) (where the cæca may take the form of two rows of short closely packed units often numbering into the hundreds), or they may be arranged in definite groups of extremely long finger-like tubes, on opposite sides of the intestine, much fewer in number than in the forms just mentioned, but compensating largely for the loss in numbers by a very marked gain in the diameter and length of the individual cæca. In all of these highly modified forms the typical arrangement is maintained. In the other group, including the Pentatomidæ, with the exception of the Asopinæ in which the cæca are absent, there are four rows of short, uniform, closely set, sac-like structures ranged along the tubular portion of the fourth stomach; and this arrangement is adhered to with remarkable uniformity in all the typical Pentatomidæ so far examined.

Before discussing the various forms of cæca in detail, it might be well to consider the probable origin and course of development of these complex organs in the different divisions of the Cimicoidea.

Recognizing the Pentatomidæ as the more primitive Heteroptera then, according to the view expressed by some authorities, the Asopinæ might be taken as representing the stock from which all the other Heteroptera developed. We find in examples of this subfamily, such as *Podisus maculiventris* (Plate II., Fig. 5), that the cæca are wholly absent, and that the fourth stomach consists merely of a short neck connecting the third stomach with the ileum. If we consider the Asopinæ as representing a type which existed previous to the first appearance of cæca in the Heteroptera, then the Thyrecoridæ should logically come next in the series, as they form a perfect connecting link, so far as the cæcal structures are concerned, between the typical Pentatomidæ with their four rows of cæca and the Lygæidæ, Coreidæ and Pyrrhocoridæ with their two rows. In this family, as represented by *Thyrecoris unicolor* (Plate I., Fig. 3), the cæca might well be regarded as showing a very primitive condition, since they appear in a double row of very blunt evaginations from the wall of the gut which shows no indication of the distinct, narrow ducts so common in most of these insects, and which might readily be imagined as in process of formation directly from folds or wrinkles in the intestinal wall. No direct transition has as yet been seen between the double row of cæca of *Thyrecoris* and the quadruple arrangement in the typical Pentatomidæ; but it can hardly be doubted that in some of the Thyrecoridæ, or perhaps in the Cydninæ, forms will be discovered in which the doubling of the rows of cæca is actually taking place. The specimens of Thyrecoris examined, and from which the drawing was made, were males; and as the cæca uniformly show a more complete development in the female than in the male, I should not be surprised to find at least indications of a third and fourth row when this sex is examined, although in the male the intestine does not show the slightest indication of this, even in sections.

This arrangement apparently does very well in the Pentatomidæ; but when the other families in which cæca occur are considered, a number of almost insurmountable difficulties are encountered.

It is easy to imagine how the complex types of cæca in the Coreidæ, Lygæidæ, and Pyrrhocoridæ could have originated from a common form like that of *Thyrecoris*, but the fact that in each of these families a number of species occur which are obviously not primitive, but in which the cæca are wholly wanting, is decidedly confusing when a progressive development of the cæca is considered.

It is probable that these organs, instead of representing a continuous line of development in the existing Heteroptera from simple to complex types, are really primitive, ancestral organs, as has been suggested by Mr. C. A. Hart, of the State Laboratory of Natural History, which are actually in process of elimination as the group advances in specialization; and that the confusing cases met with in the three families just mentioned should really be regarded as more highly specialized forms in which the cæca have entirely disappeared. This view seems to agree in essentials with the best grouping of the Heteroptera, and Mr. Hart, who has kindly compared the evidence presented by the structures with the principal systems of classifications that have been proposed for these insects, finds that the relationships indicated by these structures agree remarkably well with the grouping of the Heteroptera as proposed by the great European hemipterist, Stål, although suggesting relationships decidedly at variance with those that have been assumed by certain American workers.

The view that the complex types of cæca should be considered the more primitive is also strongly supported by embryological evidence.

Upon dissecting the embryo of the pentatomid, *Murgantia histrionica*, a day or more before time for hatching and shortly after the midgut had formed, the cæcal region was found to be represented by a comparatively long, pink section of the intestine, upon which the four rows of minute cæca were appearing, each cæcum originating as an independent evagination of the wall of the embryonic intestine.

A similar study of the embryonic gut of such Lygæidæ as *Blissus leucopterus* would be very desirable, in order to see if the typical grouping of the cæca of the Blissinæ is not merely a specialization from forms such as those in the Pachygronthinæ. An examination of the embryonic cæca of the Thyrecoridæ as well as of the Cidnidæ, would also be worth while, as it is here that we should expect the actual transition to take place from the four-rowed form of the typical Pentatomidæ to the forms with but two rows, if the cæca in the Heteroptera are really being reduced in complexity.

If this view is correct, and it can hardly be questioned, then the Asopinæ could not be considered the more primitive type of the Heteroptera, as has been suggested by Kirkaldy, but would have to be considered a much more specialized group than the Pentatominæ, in which the cæca reach their greatest development in such forms as *Brochymena quadripustula*, where there may be as many as 1,400 cæca in the entire system.

Just why the cæca should drop out so suddenly in the Asopinæ is not clear, unless this is due to a specialization of the alimentary canal correlated with the strictly predaceous habits of these insects. In this connection, it is of interest to note that the cæca are invariably absent in all the strictly predaceous Heteroptera.

The Scutellerinæ, judging from their cæcal equipment, are evidently close to the primitive Pentatominæ. Of the Cydninæ but a single male of *Amnestes pusillus* has been examined; and as the cæca were apparently absent in this specimen, this subfamily may be further removed than even the Thyrecoridæ, although no generalization can be made from this one dissection.

In regard to the function of the cæca of these insects, it can only be said that no digestive function has been discovered, and that the food mass in process of digestion apparently never gets beyond the third stomach.

This, together with the fact that these organs are located at the extreme lower limit of the digestive portion of the gut and are apparently in process of elimination, would seem to show that they possess no important digestive function, although the great development of these structures in forms like *Brochymena* would certainly suggest this.

It seems not improbable that the present function of the cæca of the Heteroptera is merely to provide a safe place for the multiplication of the normal bacteria of these insects.

It was originally planned to include in the present paper a discussion of the histology of the different divisions of the alimentary canal, as well as a comparative treatment of the cæca from the different insects studied, having in mind particularly the bearing of these structures on the relationships and classification of the different groups of Heteroptera; but owing to lack of available space this aspect of the work will have to be omitted for the present and reserved for publication at another time. A glance, however, at the accompanying illustrations of typical cæca selected from a few of the Heteroptera dissected, will show very well the range of development of these organs in this group, and also the possibilities that open up for a very thorough and complete survey of the cæca in these insects.

Some idea of the ground already covered in this phase of the work can be had from the following list of species, representatives of all of which have been dissected and studied; each species usually representing a large number of individual dissections.

It will be highly desirable to dissect a much wider range of species than is included in this list before making any final generalizations, and all alcoholic or preferably living material that any reader may be able to contribute to supplement this list, especially in the Lygæidæ or Coreidæ, will be very welcome indeed.

Many of the species already dissected were obtained through the kindness of Mr. C. A. Hart, of the State Laboratory of Natural History, to whom I am greatly indebted. I wish also to thank Mr. L. M. Smith and Mr. W. P. Flint, both of the Illinois state entomologist's office, for much valuable assistance in securing material for the work.

The following are the species that have been dissected so far in connection with this problem:

Pentatomidæ.
- *Brochymena quadripustulata* Fabr.
- *Euschistus fissilis* Uhl.
- *Euschistus servus* Say.
- *Euschistus variolarius* Pal. Beauv.
- *Murgantia histrionica* Hahn.
- *Nezara hilaris* Say.
- *Thyanta custator* Fabr.
- *Thyanta preditor* Fabr.
- *Peribalus limbolarius* Stål.
- *Hymenarcys æqualis* A. & S.
- *Œbalus pugnax* Fabr.
- *Cosmopepla carnifex* Fabr.
- *Neottiglossa undata* Say.
- *Mormidea lugens* Fabr.
- *Cœnus delius* Say.
- *Proxys punctulatus* Pal. Beauv.
- *Podops cinctipes* Say.
- *Podisus maculiventris* Say.
- *Perilloides circumcinctus* Stål.

Scutelleridæ.
- *Homoemus proteus* Stål.

Cydnidæ.
- *Amnestus puscillus* Uhl.

Thyrecoridæ.
- *Thyrecoris unicolor* Pal. Beauv.
- *Thyrecoris pulicaria* Germ.

Coreidæ.
- *Archimerus indecorus* Walk.
- *Metapodius terminalis* Dall.
- *Euthoctha goleator* Fabr.
- *Anasa tristis* De G.
- *Anasa armigera* Say.
- *Chariesterus antennator* Fabr.
- *Catorintha mendica* Stål.
- *Narnia pallidicornis* Stål.
- *Hypselonotus punctiventris* Stål.
- *Alydus conspersus* Mont.
- *Alydus quinquespinosus* Say.
- *Stachyocnemus apicalis* Dall.
- *Harmostes reflexulus* Stål.
- *Corizus lateralis* Say.
- *Leptocoris trivittatus* Say.

Pyrrhocoridæ.
- *Largus sinctus.*
- *Dysdercus suturellus* Scha.
- *Dysdercus flavolimbatus* Stål.
- *Dysdercus splendidus* Dist.

Lygæidæ.
- *Peliopelta abbreviata* Uhl.
- *Gonianotus marginepunctatus* Wolff.
- *Œdancala dorsalis* Fabr.
- *Myodocha serripes* Oliv.
- *Blissus leucopterus* Say.
- *Microtoma carbonaria* Rossi.
- *Sphragisticus nebulosus* Fall.
- *Ischnodemus falicus* Say.
- *Cymus angustatus* Stål.
- *Nysius ericæ* Schill.
- *Perigenes fallax* Heid.

Ligyrocoris diffusus Uhl.
Pamera basalis Dall.
Eremocoris ferus Say.
Ischnorhynchus didymus Zett.
Geocoris uliginosus Say.
Geocoris limbatus Stål.
Oncopeltus fascinatus Dall.
Lygæus kalmii Stål.
Lygæus bicrucis Say.

Berytidæ.
Jalysus spinosus Say.

Aradidæ.
Aradus similis Say.

Tingitidæ.
Piesma cinerea Say.
Corythucha arcuata Say.

Nabidæ.
Coriscus ferus Linn.
Coriscus pallescens Reut.

Phymatidæ.
Phymata erosa Linn.

Reduviidæ.
Melanolestes picipes H. S.
Sinea diadema Fabr.
Acholla multispinosa De G.

Emesidæ.
Emesa longipes De G.

Clinocoridæ.
Cimex lectularius Linn.

Anthocoridæ.
Triphleps insidiosus Say.

Capsidæ.
Lygus pratensis Linn.
Lopidea media Say.
Calocoris rapidus.
Hyaliodes vitipennis Say.

Notonectidæ.
Notonecta undulata Say.

Anisops platycnemis Fieb.
Nepidæ.
Ranatra quadridentata Stål.
Belostomidæ.
Benacus griseus Say.
Belostoma fluminea Say.
Veliidæ.
Hebrus americana Uhl.
Gerris marginatus Say.
Gerris regimis Say.

Functional Relation of Cæcal Bacteria to the Host Insect.

In considering the possible relation of the cæcal bacteria to the life processes of the insect, a digestive function is at once suggested on account of the great number and apparent limitation of these organisms to the digestive tract of the host. One point, however, that was rather puzzling and decidedly difficult to understand on this basis was the peculiar localization of the infection in relation to the digestive portion of the gut.

Digestive cæca are very common in other groups of insects, occurring notably in the Orthoptera, Coleoptera, and Diptera, as well as in several other orders, but with a few isolated exceptions these organs are invariably located toward the anterior end of the midgut, often serving as reservoirs for the food in which certain definite digestive processes take place. In the Heteroptera, however, the cæca, and consequently the intestinal bacteria, are located at the very posterior end of the midgut, and these organs appear to have lost their direct digestive function, since neither they nor the fourth stomach itself ever seem to contain any food, the last stages of digestion apparently taking place in the third stomach, which is usually found to contain a mass of food material in process of digestion. Just how to correlate the singular localization of the intestinal bacteria in these insects with any digestive process was by no means clear, and this remained more or less of a mystery until explained by culture experiments.

It is a well-known fact that most insects, whether feeding on solid or liquid food, support a great variety of saprophytic and parasitic bacteria and protozoa in the alimentary canal. This relation is especially marked in those insects feeding normally on solid materials which may be more or less contaminated in the beginning, but it also applies to sucking insects which feed normally on sterile liquids, although such insects are usually less heavily infected. Mosquitoes and other blood-sucking insects are excellent examples of this, for while they feed largely on the normally sterile blood of vertebrates they usually contain a great variety of bacteria and flagellate parasites in the stomach and intestine, which clearly have no relation to the vertebrate from which the food was secured.

Early in the work a peculiar infection was observed in *Peribalus limbolarius*, a pentatomid in which the cæca are well developed. In the specimens of this species, collected from certain localities, fully ten per cent. of the individuals showed an extremely heavy infection of the large sac-like reservoirs of the salivary glands (Plate II., Fig. 4) with a flagellate of the *Herpetomonas* group. This organism was apparently going through a normal developmental cycle in these organs, as all stages could be readily observed, ranging from minute, rounded or pear-shaped, non-motile bodies, with a distinct nucleus and blepharoplast, but with no flagellum, to the long, slender, typical *Herpetomonas* stage. Multiplication rosettes were also abundant, showing that the organism had evidently fully established itself in these organs. Infection with organisms very similar to the species found in *Peribalus* have been observed and described in connection with a very large number of different insects, including especially many Diptera and predaceous Heteroptera, but so far as I have been able to discover, these infections have been confined to the stomach and intestine and are never known to be localized in the salivary glands.

In working up the development of the *Herpetomonas* from *Peribalus*, the alimentary canal was searched very carefully in a large number of individuals, as it was thought that the organism observed in the salivary glands probably represented the final stage in the life history of some intestinal form; but although a very thorough search was made, not the slightest indication was found of an invasion of the midintestine by this parasite, although the salivary glands often appeared almost completely filled with them.

Some time later, upon examining specimens of *Podisus maculiventris*, an insect of the same family, but in which the cæca are wholly wanting, a similar infection was observed. This time, however, the alimentary canal was the seat of the infection; and although a very careful examination was made of the salivary glands in specimens showing a very heavy intestinal infection, these organs were found to be invariably free from any infection with the flagellate. In *Podisus* the infection appears to be remarkably common, fully fifty per cent. of the specimens from some localities containing this parasite. The third stomach seems to be the place of greatest multiplication of the flagellates in this insect, and not only the *Herpetomonas* but also various forms of foreign bacteria were observed in this region. This is in marked contrast to the condition found in the midgut of those forms which are provided with cæca; for of the hundreds of typical pentatomids examined, flagellates were found in the alimentary canal of but two specimens of *Cœnus delius*, which were apparently on the point of dying when dissected, and in these the parasites were confined chiefly to the first stomach instead of reaching their greatest development in the third stomach as in *Podisus*.

For a long time after the singular infection of the salivary glands in *Peribalus* had been observed, no satisfactory explanation could be offered for the strict localization of the typical intestinal flagellates in these organs. It was not realized at first that there might be some possible connection between this phenomenon and the bacteria normally infecting the cæca of the insect, but later when this case was compared with similar infections in such forms as *Podisus*, in which the cæca were wholly wanting, it seemed that the cæcal bacteria must in some way be responsible, and this view was later confirmed by direct culture experiments.

We may assume that these *Herpetomonas* parasites, upon being continually introduced into the alimentary canal of *Peribalus*, and being unable to develop in the midgut in the presence of the antagonistic cæcal bacteria, gradually become adapted to a life in the bag-like salivary reservoirs instead of being excluded entirely as was apparently the case in most other Heteroptera in which the cæca are present.

As a result of the apparent failure in the culture work with *Murgantia* and the other Pentatomidæ followed by the successful cultivation of the cæcal bacteria from *Anasa tristis*, it became evident that at least one perfectly clear-cut function possessed by these organisms, whether of profound importance to the host insect or not, was the antagonism which they certainly show towards other bacteria and protozoan parasites which would normally be expected to occur in the intestine of such insects.

In the culture work on the Pentatomidæ it was found that the entire cæcal system of one of these insects could be removed and dropped directly into a tube of bouillon or other media, where it would remain for a month or more without a trace of growth developing. This was not an occasional occurrence, but was invariably the result secured where the Pentatomidæ were used and demonstrated conclusively that the cæcal bacteria are not only antagonistic to the ordinary saprophytic and parasitic bacteria, but prevent their development entirely, and apparently kill them when they invade the alimentary canal of these insects.

In the somewhat similar association described by Petri for the larva of *Dacus oleæ* the bacillus concerned was found to secrete an active lipolytic enzyme which presumably assisted the insect in digesting its oily food, but unfortunately Petri apparently did not consider the possible antagonism of these cæcal bacteria of the fly toward the forms which commonly invade the alimentary canal of such insects; and it is consequently impossible to say whether the cæcal bacteria of the little fly resemble those of the Heteroptera in this regard or not.

In regard to a digestive function for the cæcal bacteria of the Heteroptera, it can only be said that in cultures these bacteria apparently secrete no enzyme that could be of any very evident assistance in the digestive processes of the insect, and this agrees perfectly with the peculiar localization of these organisms at the extreme posterior end of the digestive portion of the gut, which in itself would render the probability of their assuming an important part in the digestion of the insect extremely small, even though they had been found to secrete important digestive enzymes.

The deficiency in enzyme production shown by these organisms will be taken up in connection with the culture work, and will not be discussed here; although it might be mentioned that when the cæca, together with a considerable section of the intestine was removed, as in the Pentatomidæ, and dropped into a tube of bouillon, the tissues would remain white and apparently normal in every way for several weeks, or longer, although crammed full of the cæcal bacteria. This would show, at least, that the cæcal bacteria do not secrete a proteolytic enzyme. The same thing was also observed in cultures from *Anasa tristis*, the tissues remaining unchanged even after the cultures had been growing vigorously for a week or more.

In the case of certain of the intestinal bacteria of the higher vertebrates, there is a well-known association existing between these organisms and the host that is remarkably similar in many ways to the one just described for the Heteroptera, although as might be expected, it is complicated, in this case, by a vast number of factors that do not have to be taken into consideration in a treatment of this relation in the much more simple insects.

Practically all the higher animals harbor certain varieties of intestinal bacteria which have become so intimately associated with the host that they are generally referred to as the "normal" intestinal bacteria; and there has been a tremendous amount of work done in the attempt to determine the exact functional importance of these organisms from the standpoint of the host. As might be expected, by far the greater part of this work deals directly with those forms peculiar to man and the higher vertebrates, but as many of the principles which have been worked out for the association in these animals throw a great deal of light on conditions as they exist in the Heteroptera, the following very brief summary of the pertinent work that has been done on this subject should not be out of place.

IMPORTANCE OF THE "NORMAL" INTESTINAL BACTERIA TO THE HOST.

The fact that certain specific bacteria occur with remarkable regularity in the complex intestinal flora of the higher animals was early recognized by bacteriologists, and this knowledge naturally led up to an extended discussion concerning the true relation of these "normal" intestine bacteria to the animal harboring them.

As early as 1885 Duclaux, by a simple experiment, established the fact that the higher plants are unable, in themselves, to utilize the more complex forms of nitrogen and that they are absolutely dependent upon the action of certain classes of bacteria normally present in the soil for their supply of this essential element of plant food.

In the same year, in commenting upon the results secured by Duclaux for plants, Pasteur gives with characteristic directness his views concerning the relation of intestinal bacteria to the assimilation of higher animals. In the following well-known quotation he says:

"Souvent, dans nos causeries du laboratoire, depuis bien des années, j'ai parlé aux jeunes savants qui m'entouraient, de l'intérêt qu'il y aurait à nourrir un jeune animal (lapin, cobaye, chien, poulet), dès sa naissance, avec des matières nutritives *pures*. Par cette dernière expression, j'entends désigner des produits alimentaires qu'on priverait artificiellement et complètement des microbes communs.

"Sans rien voulair affirmer, je ne cache pas que j'entreprendrais cette étude, si j'en avais le temps, avec la pensée préconçue que la vie, dans ces conditions deviendrait impossible."

Although the above hypothesis has excited a vast amount of discussion, and although a number of very elaborate experiments have been carried out to test it, the exact relation of the "normal" intestinal bacteria to the animal harboring them is, as yet, by no means definitely settled. Thus, some authorities advance the idea that these bacteria are merely saprophytic forms which, through long association, have become adjusted to conditions in the digestive tract where they exist without exerting any important influence upon the host, unless perhaps in exceptional cases, when they may assume the rôle of active parasites. Others hold, on the contrary, that their presence is of very great importance in the overgrowing and destroying of the occasional invader, which if allowed to develop unchecked might seriously injure the host, and also in the inhibitory action which they exert upon the common putrefactive intestinal bacteria; while still others assert that, in addition to the function just mentioned, these bacteria not only play an important part in, but are absolutely essential to proper digestion, at least in the higher animals.

The first serious attempt to determine by actual experiment the part played by intestinal bacteria in digestion appears to have been made by Nuttall and Thierfelder in their classical experiments with guinea pigs. In these experiments the young animals were removed from the mother by Cæsarian section and transferred at once to ingeniously constructed cages where they were kept under absolutely aseptic conditions and supplied with sterile food and water. Under these conditions the authors were able to keep the animals alive for ten days, at the end of which time they were found to have increased considerably in weight. Of the four animals carried through the experiment the increase in weight was found to be 5.5, 14, 16 and 28 grams respectively.

From their data the authors conclude that intestinal bacteria are not essential to digestion and their results have been widely quoted by physiologists as showing that the intestinal bacteria are at most of only minor importance in this process.

The results of these experiments are far from being conclusive. as has frequently been pointed out. Thus, Schottelius was able to keep newborn guinea pigs alive for ten days by giving them nothing but sterile water. The same author also found that the intestinal contents of a normal ten-days-old guinea pig weighed from twelve to fifteen grams, and in this case the intestine was not completely filled; while Nuttall and Thierfelder, upon examining the pigs at the conclusion of their experiments, found that the colon and especially the cæcum were crammed full, and even greatly distended, with a brown caseous material. Schottelius insists that this constantly accumulating mass of undigested food in the intestine would much more than account for the increase in weight reported by Nuttall and Thierfelder and that, instead of increasing in weight, their animals must have been actually starving because unable in the absence of the proper intestinal bacteria to digest the food swallowed.

Pasteur's hypothesis as to the function of intestinal bacteria was, a few years later, again tested experimentally by Schottelius, who, after carrying out a carefully planned series of experiments, arrived at conclusions decidedly different from those of Nuttall and Thierfelder.

In the experiments of Schottelius chickens were used instead of guinea pigs. The artificially incubated eggs were removed and carefully sterilized shortly before the time for hatching and placed for hatching in a specially constructed, sterilized cage, together with sufficient sterile food and water to last throughout the course of the experiment. It was found that chicks hatched and reared in this way, with the total exclusion of bacteria, showed but very little growth; and while they continued to eat ravenously, being apparently unable to satisfy their hunger, they gradually became weaker and after an apparent slight increase for the first few days invariably died, usually within twenty-five days or less after hatching.

In the control cages, which contained chicks treated in exactly the same way except for the addition of a pure culture of a colon bacillus to the food, the young animals developed normally in every way and even appeared to show slightly better growth than chicks of the same age which had been allowed to run free in the laboratory as a counter check.

These results are regarded by the author as showing conclusively that intestinal bacteria, especially of the *Bacillus coli* type, are absolutely essential to proper digestion in chickens and that they doubtless have a similar function in other animals. He summarizes as follows the precise manner in which he thinks the normal bacteria influence the host:

"1. Die Darmbakterien sind notwendig für die Ernährung der Wirbeltiere und für den Menschen;

"2. Der Nutzen der normalen Darmbakterien besteht: (*a*) in der Vorbereitung der Ingesta für die Resorption der Nahrungsstoffe; (*b*) in der Reizung der Darmwand zur Auslösung der Peristaltik; (*c*) in der Überwucherung und Vermichtung pathogener, in den Darm hineingelangter Bakterien; (*d*) in der Festigung des Körpers gegen pathogene Bakterien und gegen Bakteriengifte."

Similar experiments with the same end in view have been carried out by Moro and by Mme. O. Metschnikoff on newly hatched tadpoles, Moro working with *Pelobates fuscus* and Mme. Metschnikoff with *Rana temporaria.* The results secured by these two workers seem to point to some important digestive function for the intestinal bacteria, although the results in neither case appear so conclusive as those given by Schottelius for his work with chicks. In the work of Metschnikoff, for example, the nearly mature embryos were removed aseptically from the gelatinous, enveloping layer and allowed to develop in vessels of sterile water under aseptic conditions. Of forty-nine tadpoles which survived the first few days of the experiment, forty-two developed accidental contamination, and seven remained sterile throughout the experiment.

Although for some reason the mortality was much higher among the non-sterile tadpoles, and although the sterile ones actually lived longest, the development of the sterile ones was much slower than that of the non-sterile, the minimum weight and length of the non-sterile corresponding closely to the maximum of the sterile. These results, however, are regarded by the author as sufficiently conclusive to warrant the assertion that intestinal bacteria are necessary for life and development of tadpoles.

Cohendy, on the other hand, in duplicating the work of Schottelius on sterile reared chicks secured results which he regards as proving conclusively that bacteria are in no way necessary to the normal development of the chicken; and he accounts for the results of his experiments as contrasted with those of Schottelius as being due to the superior technique which he claims to have employed. In the course of this work he was able to keep chicks under strictly aseptic conditions for a maximum of forty days during which time the animals developed normally, there being no essential difference in growth and metabolism between the sterile chicks and those kept as a check, and the sterile chicks when placed under natural conditions, after having been kept wholly free from bacteria for a considerable time, developed into perfectly normal adults. His conclusions, which are quite different from those of Schottelius, are: "La vie sans microbe est possible pour un vertébré—le poulet—pourvu normalement d'une riche flore microbienne. Cette vie aceptique n'entraîne par elle-même aucune déchéance de l'organisme."

Insects and other invertebrates have been employed by a number of investigators, as Bogdanow, Woolman, and Delcourt and Guyenot, for working out this same problem. Of these the work of Delcourt and Guyenot is especially notable, for while no vertebrate has, so far, been carried successfully through its normal life cycle in the total absence of intestinal bacteria, these authors have succeeded in keeping flies of the genus *Drosophila* for as many as twenty generations under aseptic conditions, finding that growth in the sterile cages was perfectly normal and fully as rapid as in similar but contaminated cages. It also developed that the mortality was remarkably reduced, the eggs almost all hatching from the sterile flies, while in the septic cages whole broods would frequently die.

Bogdanow and Woolman, who also worked with flies, chiefly *Lucilia cæsar* and *Calliphora vomitoria*, both succeeded in rearing these insects with the exclusion of bacteria, but their conclusions as to the importance of intestinal bacteria in growth and development are somewhat different. Bogdanow found that, while the larvæ would develop under these conditions, growth was greatly retarded, and practically all died at or before pupation although one would occasionally pupate and develop into an undersized fly. He concludes that his experiments show that bacteria, probably those species which decompose protein, are necessary to the normal development of these flies.

Woolman, however, was able to carry the larvæ through from sterile eggs to normal adults. During the first few days after hatching the growth of the sterile maggots was noticeably slower than in the septic cages, but later this difference tended to disappear, and when mature the sterile larvæ showed practically the length and weight typical for the species, pupated, and developed into perfectly normal adults. This slight check in growth shown by the maggots at first was attributed by the author to the sterilization of the media at too high a temperature, as it was found that the difference in rate of development largely disappeared when the food was sterilized by the discontinuous method at low temperatures. Maggots hatched from sterile eggs were also treated with cultures of various bacteria, including *Bacillus coli*, *Bacillus proteus vulgaris*, *Bacillus putrificus*, and others, in order to determine the part taken by proteolytic bacteria in development, as suggested by Bogdanow, but the infected larvæ developed no more rapidly than the sterile ones and where *Bacillus putrificus* was used growth was clearly retarded and the larvæ died regularly before reaching the pupa stage. Woolman concludes that: "Cet example d'un être qui, à l'état naturel, semble vivre en association étroite avec les bactéries, montre clairement que la vie animale est possible en dehors de toute intervention des microorganismes."

It can hardly be doubted that many insects, such as those living parasitically in the body cavity of the host, normally exist during the greater part of their life cycle in the total absence of bacteria; and according to Portier this is also true of the larvæ of certain leaf-mining microlepidoptera. This author found that about 30 per cent. of the *Lithocolletis* larvæ infesting oak, elm, etc., were wholly free from bacteria and that practically 100 per cent. of the larvæ of a species of *Nepticula* infesting the rose and which do not void the excrement on the exterior of the mine were also sterile.

Notwithstanding the direct experimental evidence in support of a digestive function for intestinal bacteria, as advanced by Schottelius and others, the view appears to be generally held by physiologists, largely on account of the characteristic limitation of the different classes of intestinal bacteria to certain well-defined regions of the digestive tract, that in the higher animals at least, the actual solution of the nutrient materials in the food, in the absorptive portion of the gut, is performed chiefly if not entirely by the digestive secretion produced by the animal itself and that the chief function of the "normal" intestinal bacteria consists, not in any direct action on the food in preparing it for resorption, but rather in preventing indirectly the undue multiplication of injurious forms which are continually invading the intestine of the host.

There appears to be little question that the normal intestinal bacteria of higher animals, such as the groups represented by *Bacillus coli*, *Bacillus* (*lactis*) *aerogenes*, and *Bacillus bifidus*, do possess definite antagonistic properties for the less perfectly adapted species, although the exact mechanism of this antagonism has been explained in a number of different ways. Some would account for this phenomenon as being due largely to overcrowding, with consequent starvation of the less hardy species. The normal forms being constantly present in the intestine in large numbers, and well adapted to vigorous growth in certain well-defined regions, would naturally be better able to appropriate any nutrient materials which had escaped resorption by the host than those forms which only occasionally invade the intestine. The various products of fermentation produced by these bacteria, especially the organic acids, are also held to be of very great importance in this same connection.

Another and apparently an exceedingly important factor in this antagonism lies in the production by these organisms of definite toxin-like bodies, the "autotoxins" of Conradi and Kurpjuweit, which in some way clearly exert a restraining influence upon the development of many bacterial species.

These latter substances, which have been carefully studied especially for *Bacillus coli*, by Eijkman, as well as by Conradi and Kurpjuweit and others, are regarded by these authors as mainly responsible for the familiar weakening and ultimate death of bacteria in old cultures which was formerly held to be due chiefly to a gradual exhaustion of the nutrient materials, resulting in death by starvation.

It was found by Eijkman, and later by Conradi and Kurpjuweit, that such an old and apparently exhausted culture could again be made to support growth by inactivating the toxins, as by heating to 60° C. or by filtering through porcelain, and that these same toxins were constantly present in normal feces, where they could be detected in very high dilutions, even as great as 1–10,000.

These "autotoxins," as the name implies, affect the organism producing them as well as various other species, although, as is suggested by Conradi and Kurpjuweit, under normal conditions in the intestine the obligate species, as *Bacillus coli*, would doubtless develop a certain amount of immunity to their own poisons which would not be shared by other transient forms.

According to Kohlbrugge there is an "autosterilization" of the small intestine in man and the higher animals generally; and normally the bacteria which occur in this part of the digestive tract are found only in the food masses, so that when the intestine is emptied the bacterial flora consequently disappears, leaving the empty intestine practically sterile. He also found that even after prolonged fasting the cæcum, that portion of the digestive tract characterized by the greatest development of bacteria of the *Bacillus coli* type, always contained quantities of this organism; and he insists that the cæcum and the vermiform appendix instead of being the useless, vestigial organs that they are commonly considered, are in reality of the greatest importance to the animal possessing them in functioning as a natural and safe culture place for a constant reserve supply of the colon bacillus.

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EXPLANATION OF PLATES.

ABBREVIATIONS.

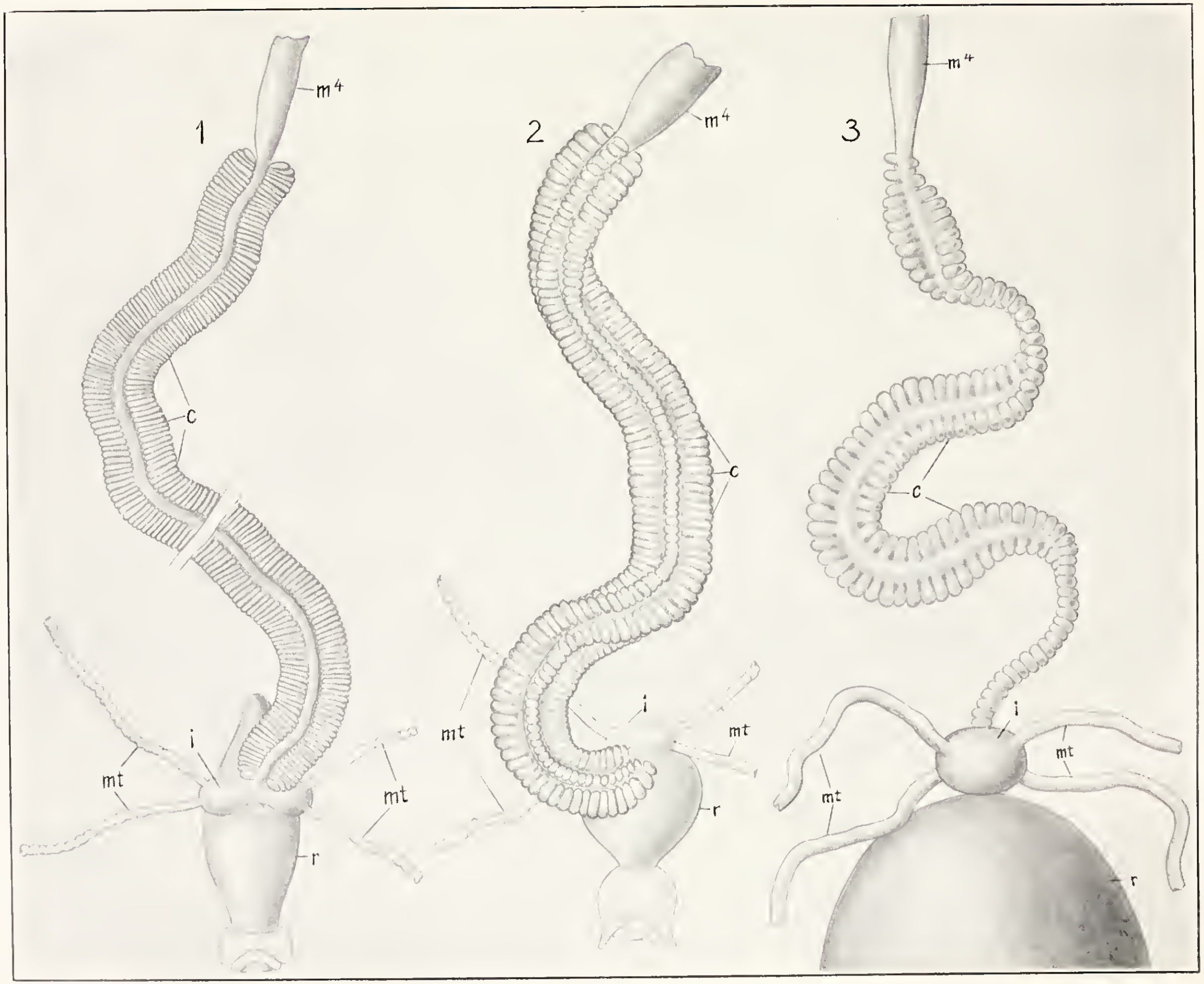
c	cæca.
i	ileum.
*m*1	first stomach.
*m*2	second stomach.
*m*3	third stomach.
*m*4	fourth stomach.
mt	malpighian tubes.
r	rectum.
sr	salivary reservoir.
sg	salivary glands.

PLATE I.

FIG. 1. *Anasa tristis*, posterior portion of alimentary canal with cæca.

FIG. 2. *Peribalus limbolarius*, posterior portion of alimentary canal with cæca

FIG. 3. *Thyrecoris unicolor*, posterior portion of alimentary canal with cæca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mt
mt
r

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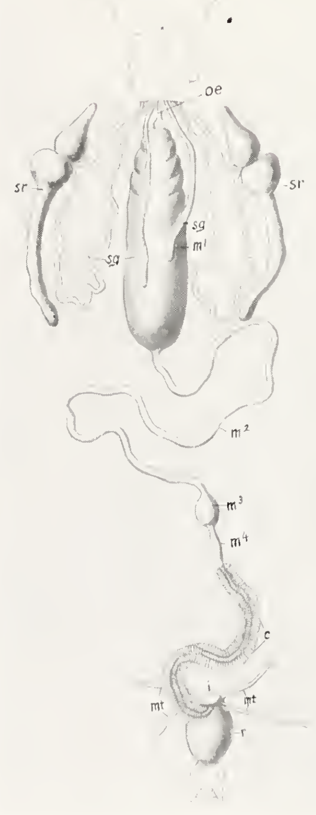
PLATE II.

FIG. 4. *Peribalus limbolarius*, entire alimentary canal with salivary glands.
FIG. 5. *Podisus maculiventris*, entire alimentary canal.
FIG. 6. *Homoemus proteus*, entire alimentary canal.

4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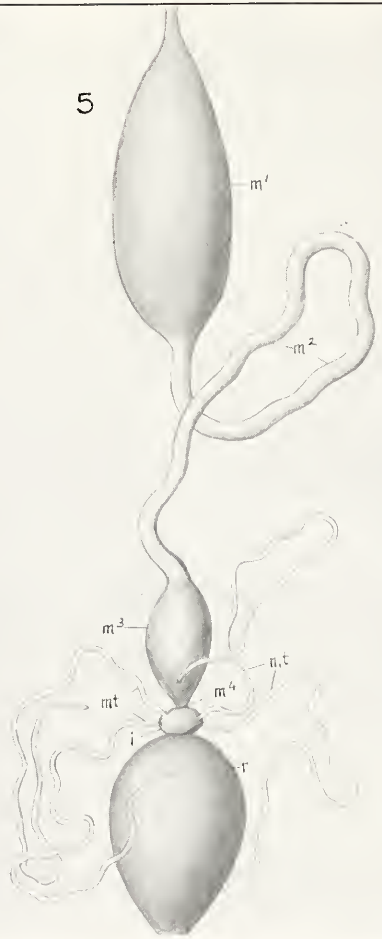
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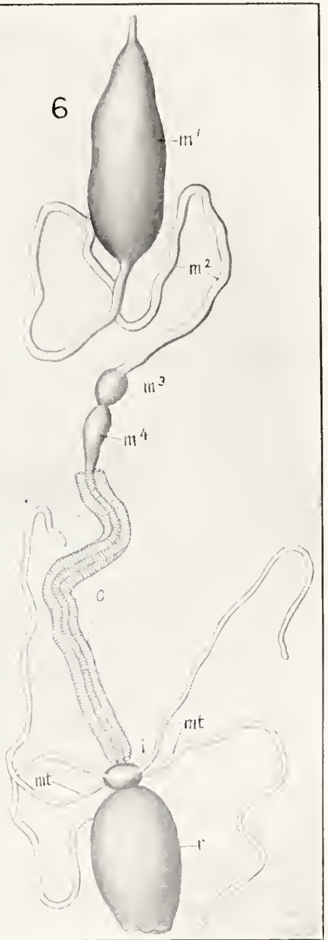
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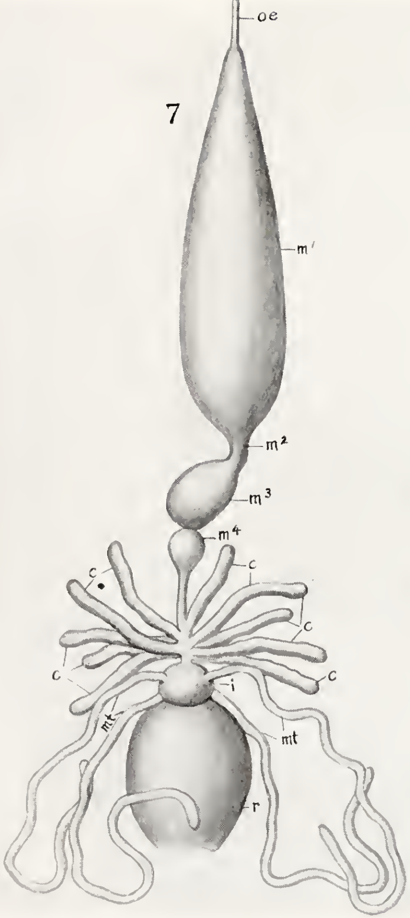
PLATE III.

FIG. 7. *Blissus leucopterus*, entire alimentary canal.

FIG. 8. *Œdancala dorsalis*, posterior portion of alimentary canal with cæca.

FIG. 9. *Myodocha serripes*, posterior portion of alimentary canal with cæca.

7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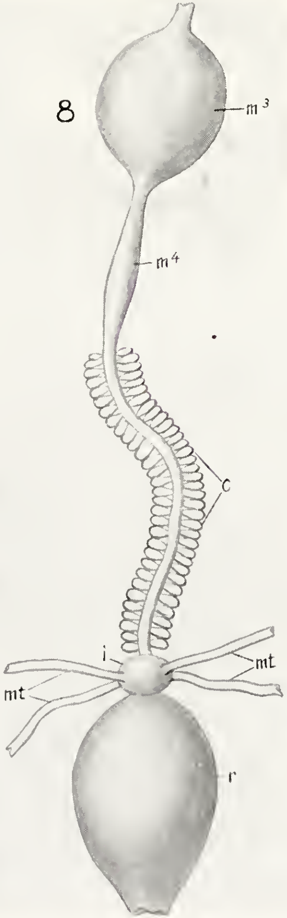
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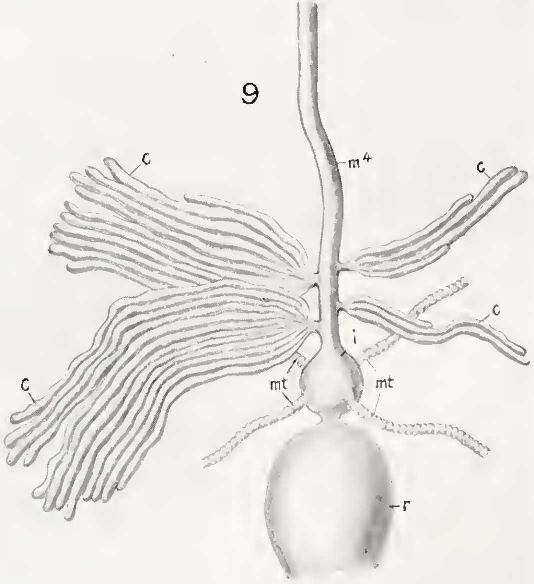
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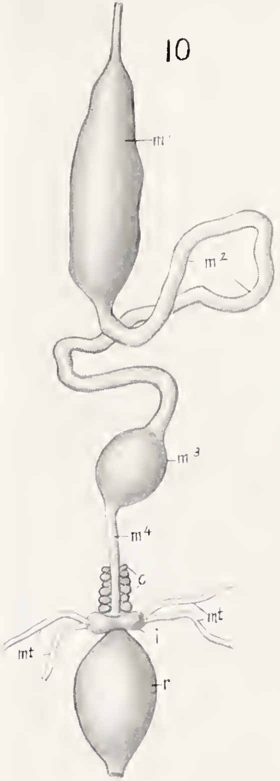
PLATE IV.

FIG. 10. *Dysdercus suturellus*, entire alimentary canal of female.

FIG. 11. *Peliopelta abbreviata*, entire alimentary canal of male.

FIG. 12. *Peliopelta abbreviata*, entire alimentary canal of female showing pair of large accessory cæca that are not present in the male.

10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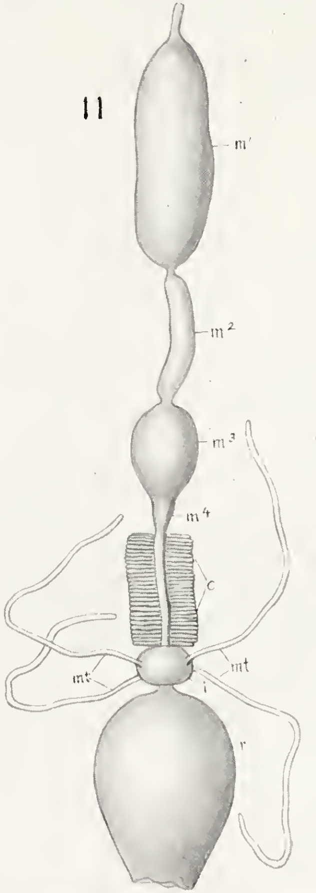
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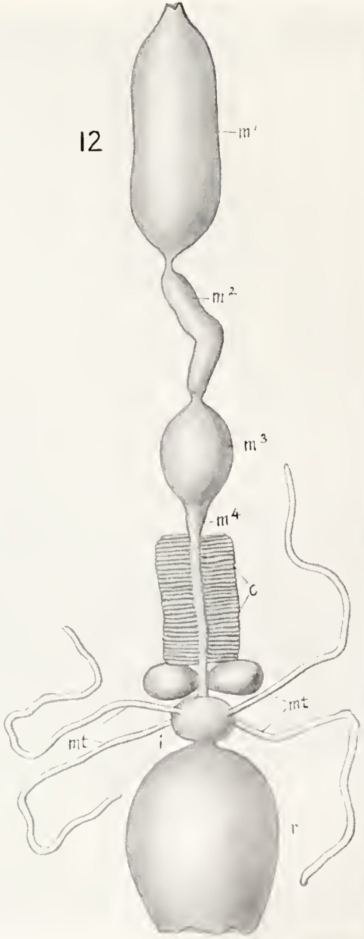
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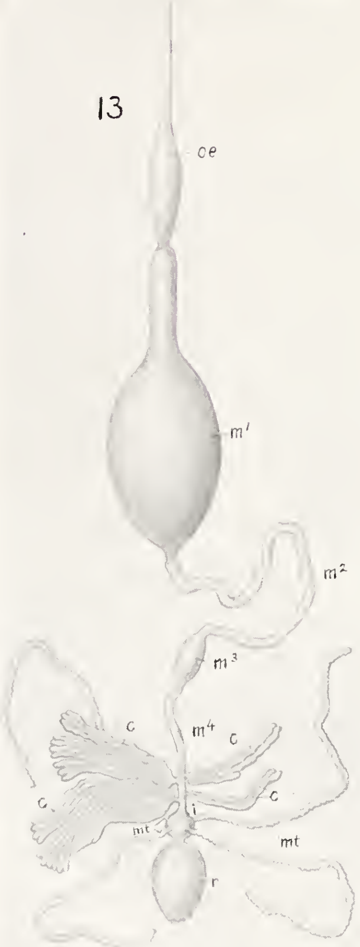
PLATE V.

FIG. 13. *Myodocha serripes*, entire alimentary canal.
FIG. 14. *Pamera basalis*, entire alimentary canal.
FIG. 15. *Jalysus spinosus*, entire alimentary canal.

13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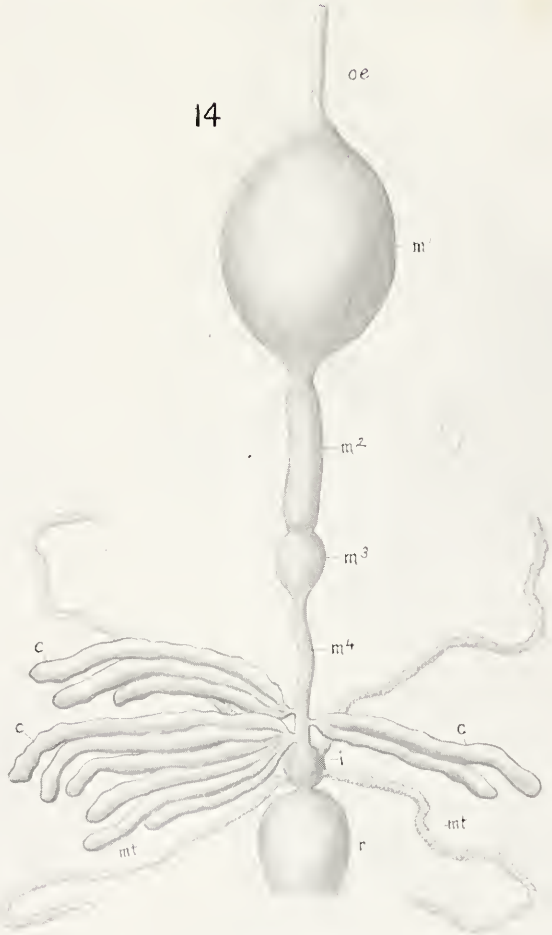
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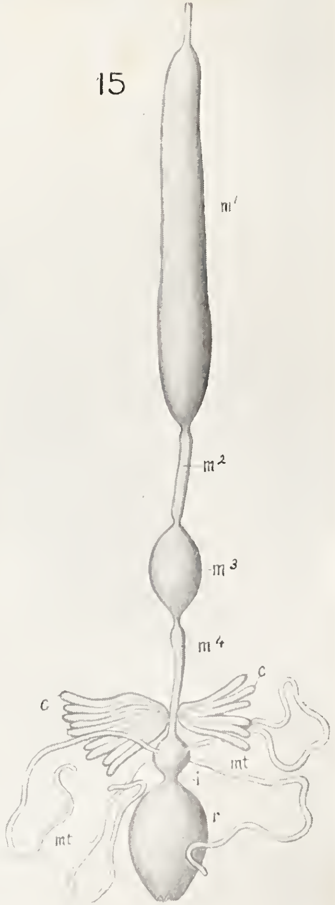
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Plate VI.

Fig. 16. *Largus sinctus*, posterior portion of alimentary canal with cæca.

Fig. 17. *Dysdercus suturellus*, posterior portion of alimentary canal of male, showing total absence of cæca in this sex.

Fig. 18. *Dysdercus suturellus*, posterior portion of alimentary canal in female showing development of the cæca in this sex.

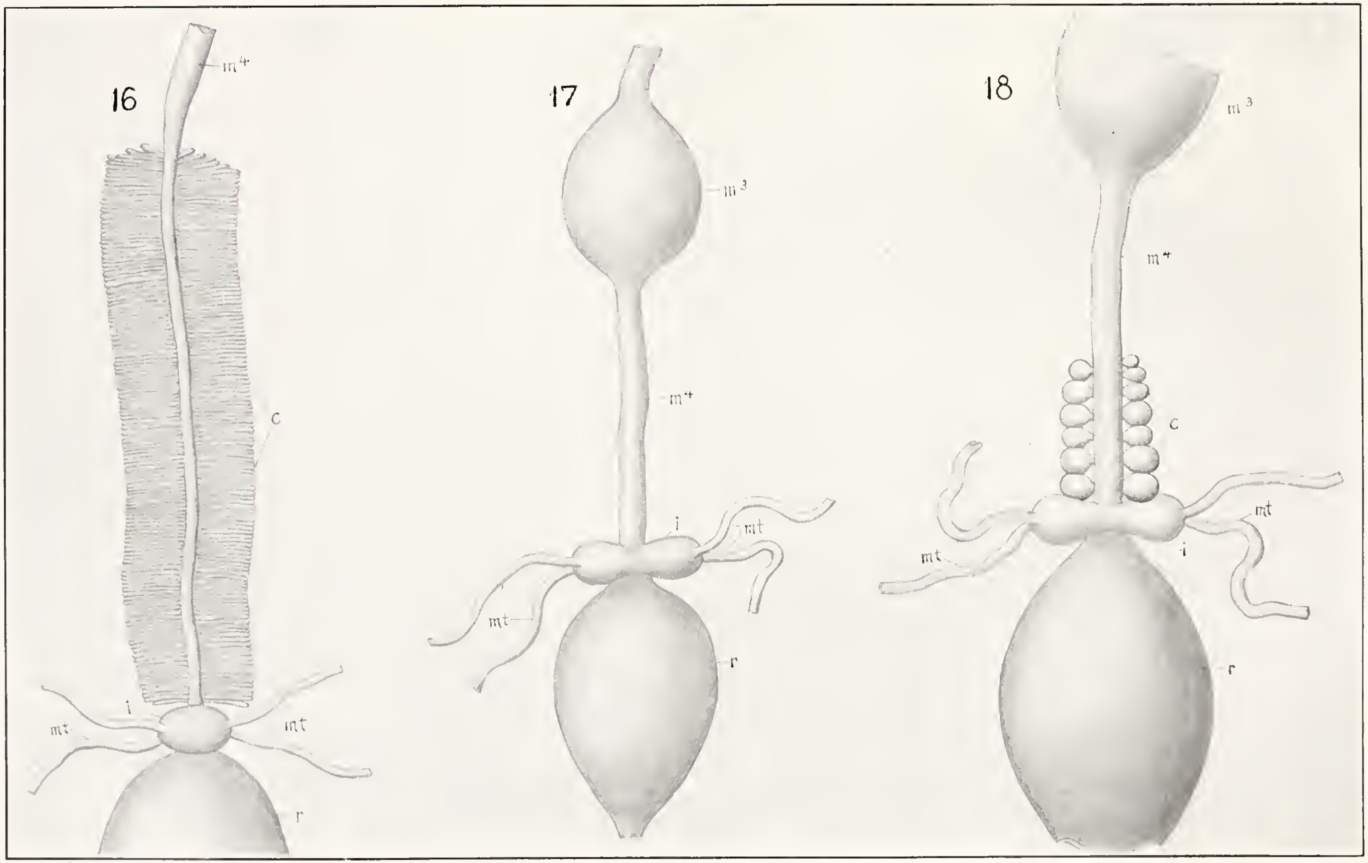
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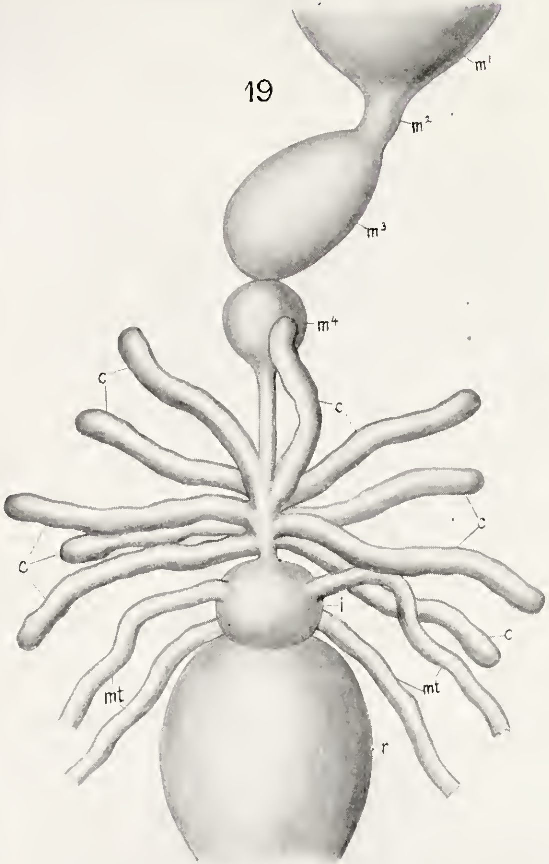
Plate VII.

Fig. 19. *Blissus leucopterus*, posterior portion of alimentary canal from normal insect showing the characteristic development of the cæca.

Fig. 20. *Blissus leucopterus*, posterior portion of alimentary canal from starved individual, the intestine also slightly stretched to show the grouping of these structures typical of the Blissinæ.

19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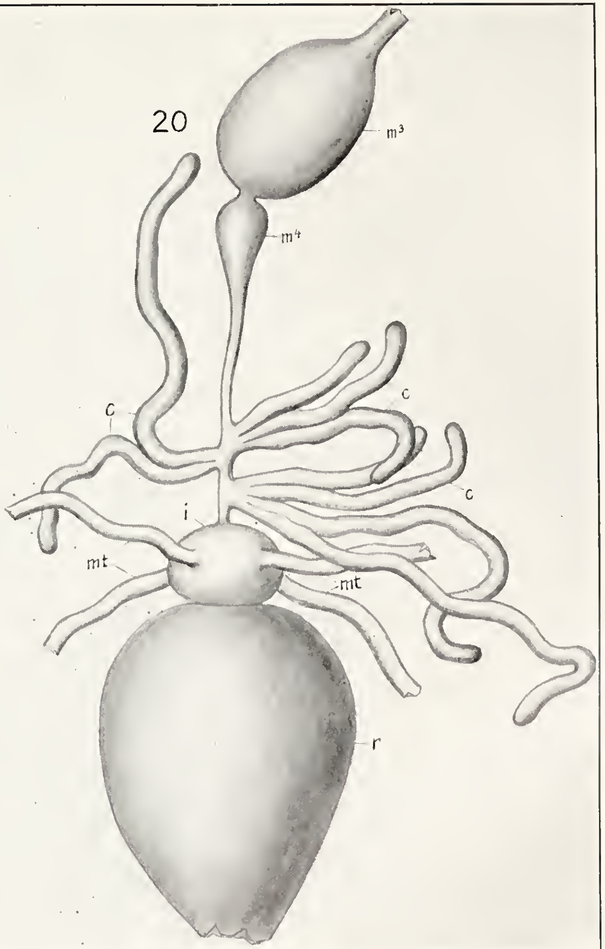
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Plate VIII.

These drawings, with the exception of Fig. 22, were all made from methylene blue smear preparations direct from the cæca of the particular host insects given. the purpose being merely to show the relative size and form of a few of the different types of cæcal bacteria.

Fig. 21. Bacteria from cæca of *Anasa tristis*. Average size of individual rods 1 by 0.7 micron.

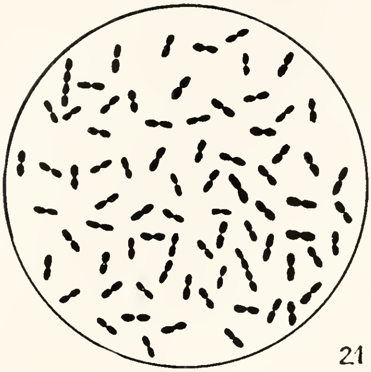
Fig. 22. Bouillon culture from cæca of *Anasa tristis*, several days old, showing common involution forms regularly produced in this medium.

Fig. 23. Bacteria from cæca of *Blissus leucopterus*. Average size of individual rods, 1 by 0.8 micron.

Fig. 24. Bacteria from cæca of *Euschistus servus*. Average size of rods, 4 by 0.9 micron, longest shown is 8 microns.

Fig. 25. Bacteria from cæca of *Peribalus limbolarius*. Size of rods, from 5 to 50 microns long by 1.2 microns.

Fig. 26. Organism from *Murgantia histrionica*. Vary from 3 to 100 microns long by 1 to 3 microns.



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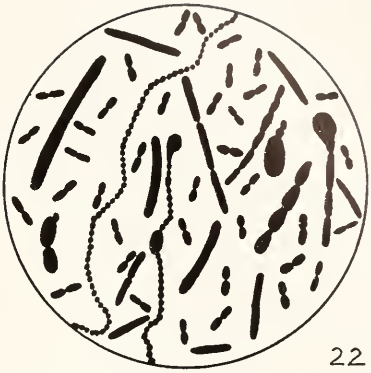

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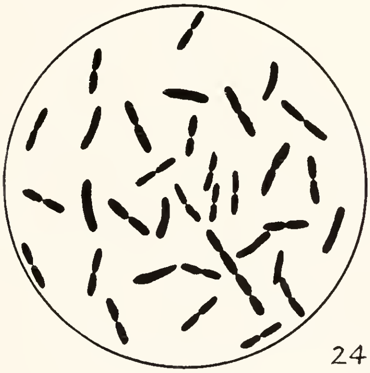

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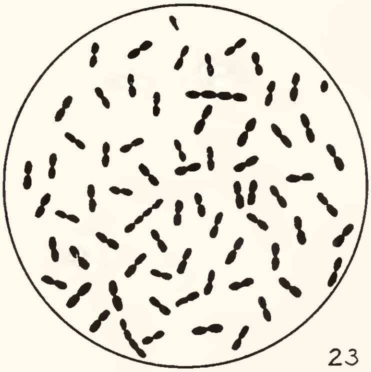

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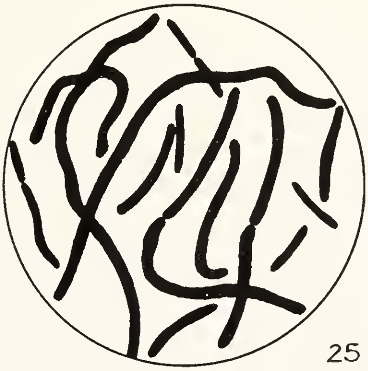

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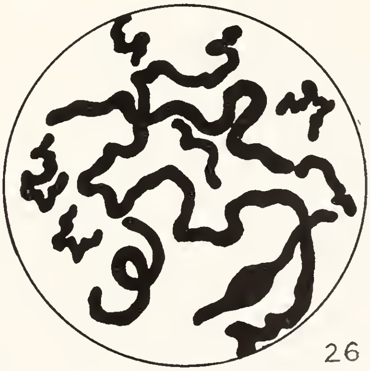

26

H GLASGOW

SEX RECOGNITION AND THE MATING BEHAVIOR OF THE WOOD FROG, RANA SYLVATICA.

ARTHUR M. BANTA.

During the last three seasons while collecting amphibian eggs for use in some experimental work the writer had opportunity incidentally to observe some interesting behavior of the wood frog, *Rana sylvatica*. Inasmuch as the mating behavior and sex-recognition of this frog appear not to have been described in any detail it seems worth while to publish a digest of the rather extensive notes made during the times of observation.

Miss Hinckley[1] has reported some observations on the egg laying and incidentally refers to the activity and "quacks" or croaks of the males at the mating season. She gives some interesting temperature observations, stating that when the air temperature is 45° F. there is little activity of the frogs but that they float on the surface "like dead leaves," but that they spawn at 50° and that at 52° they are "active and clamorous." The mating season evidently begins earlier at Milton, Mass., where her observations were made, than at Cold Spring Harbor, for in 1880 she observed the frogs at ponds on Feb. 28 and in 1881 saw eggs and the quacking males on March 8.

Wright[2] describes the appearance of the eggs and gives the season at Ithaca, N. Y., as April 1 to 30.

The pond at which the writer mainly observed these frogs is perhaps 100 by 40 feet. It was formerly part of an artificial lake from which it was cut off by a grade intended for a railway. In the years since the grade has been abandoned the "Cut-off Pond" has become much filled up with leaves and other debris, so that now it is shallow, largely filled with leaves and much encroached upon about the margin.

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[1] Mary H. Hinckley, "Notes on the Development of *Rana sylvatica* Leconte," *Proc. Bost. Soc. Nat. Hist.*, Vol. 22, pp. 85–95, 1882.

[2] Albert Hazen Wright, "The Anura of Ithaca, N. Y.: A Key to their Eggs," BIOL. BULL., Vol. 18, pp. 69–71, 1910.

The frogs were first observed in the breeding season in 1911 when on approaching the pond (March 30) at 100 yards' distance the writer heard a chorus of peculiar quawks. On nearing the pond the surface was seen to be constantly agitated and rippled in many places simultaneously by the movements of scores of frogs.

There have been 150 to 250 of these frogs at the Cut-off Pond each of the past three breeding seasons. The males lie on the surface of the water with outstretched legs each one quawking and swimming about at frequent intervals. The croak or short quawk differs decidedly from the croak of other frogs known to the writer. The note is not a distinct croak nor a peep but is a somewhat guttural, though not a coarse tone, perhaps slightly resembling the quack of a duck but more like the quawk of the night-heron. But it is a shorter note; it is not so loud and is less bird-like and more frog-like than the night-heron's call. The frogs have a single quawk or croak usually not repeated for some little interval. It is produced occasionally by the pairing males and frequently by the single males while lying quietly on the surface or resting partly upon a piece of brush or other debris. There is also a series of notes rather less loud and in slightly higher tone than the single call. This series is emitted by the males while swimming with a series of short, very rapid, leap-like strokes, a note accompanying each extension of the hind legs when the frog begins to swim. It is also sometimes made by a pairing male when with its mate it swims at the surface. Usually each successive note is less loud and each swimming movement less vigorous than the preceding one. The swimming movements of a series often continue after the calls cease. The series of calls, as well as the single note, when uttered by the pairing males is somewhat modified (apparently by the contact of the male's throat with the female's body) so that one can often distinguish the call of the pairing from that of the single male.

In swimming the head is held well out of the water and each stroke tends to push the frog more or less above the surface. The strokes are repeated in such quick succession that little advantage is obtained from the momentum acquired from the previous strokes and the tendency to push out of the water is such that the progress made is relatively small. At the end of the series of swimming movements the hind legs are often vigorously extended alternately thus swaying or turning the body from side to side but resulting in very little forward movement. The series of short swimming movements are repeated by the individual at varying intervals from a few seconds to several minutes. When the activity is at its height few of the males remain inactive for as long as a half minute at a time and the calls from the numerous males make an interesting and peculiar and not unattractive chorus. The swimming movements are associated with seeking a mate. At the height of the chorus the frogs present a picture of remarkable activity for amphibians, the males swimming about and each attempting to mate with any frog or small moving object it encounters. Any individual which moves within a radius of several feet of another male is likely to be tested by him. The male thus approached sometimes swims away and sometimes actively resists but often pays no attention to the aggressor and the latter turns back, frequently without coming near enough to touch the male, and almost always the aggressor gives up the attack after the very beginning of an attempt to grasp the stranger with the fore legs. Often the one just attacked turns and "tries" the one that has just then given up an attack upon him but with an equally prompt cessation of the attempt. Seldom is an attempt made upon any but a moving individual. Even a female in the midst of a number of males may usually avoid pursuit as long as she remains quiet. On the other hand any small moving object at the surface of the water is most certain to be approached by an eager male. The writer twice observed a male approach a speckled tortoise when the latter thrust its head out of the water.[1]

The mating activities of the male frogs are not very readily interrupted. By moving quite slowly one may ordinarily approach to within a few feet of the active males without disturbing their chorus. When persons passed noisily by on the old grade however the chorus was more or less quieted, often stopped entirely, and if the disturbance was considerable the males would leave the surface of the water. The renewal of the chorus was then uncertain. It might be renewed soon or not for an hour or two or if the air became cooler in the meantime it might not be heard again that day. The amount of disturbance required to interfere with their activities depended upon the degree of excitement of the chorus, and upon whether or not there had been any previous disturbance. Several times upon my approach to the pond during the breeding season a Cooper's or sharp-shinned hawk flew from a tree immediately over the pond. Few frogs were seen at the surface and these had only their heads protruding and many of them disappeared at the slightest unusual movement in the neighborhood. There seemed good reason to think that the hawk had been feeding upon the frogs, for it was seen there often and each time the frogs were less in evidence than otherwise noted during the breeding season when conditions were at all favorable for their seeking mates. At one time when the writer was at the pond a crow flew over casting a shadow across the pond and the chorus quickly died down and almost stopped but there being no further alarm it came up again at once. The following note was made after a hawk had been frightened away from the pond when the frogs had been much disturbed and the pond seemed almost destitute of frogs: "*Very* few frogs visible. 15 minutes later one of the decoys in the bag croaked,—a submerged almost choked-off croak. It was followed soon by two or three other croaks from the same place and then one of those in the pond uttered a single croak and, the wave of confidence spreading, in two minutes the chorus was in full swing and the pond where all had been still as death was filled with active croaking tackling frogs." Such a speedy renewal of the chorus seems to indicate excellent perception on the part of the submerged males of the first sounds made by the few males at the surface.

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[1] One suspects Miss Hinckley may have mistaken the mating activities of the males for she says (*l.c.*, p. 88): The ". . . presence of the females, who were largely outnumbered by the other sex, had evidently aroused a spirit of jealously among them and each frog was intent on driving the others from the place."

The males remain at the surface at night when the air is not too cool and on one moonlight night the full chorus was observed at 10 P.M.

The behavior of the female wood frog is usually very different from that of the male. The female remains at the bottom of the pond or clings to debris well beneath the surface and comes to the surface only occasionally. Even when unobserved by a male the female apparently does not remain at the surface for more than a few seconds at a time. When the female comes up she usually dodges under again at once to avoid the approach of one or more males. Sometimes she swims for a short distance on the surface and then goes under but more usually she dodges under at once and either remains beneath the surface or after swimming with a few long effective strokes comes up again some little distance away. The males follow a disturbance of the surface almost as readily as the moving object itself. If a female, though submerged, swims near enough the surface to produce some little ruffling of it, one or more males is very likely to follow the disturbance and attempt to seize the female when she comes up. Once in a while the female leaves the water and with long leaps moves about over the bank near the level of the water. She (as well as any male moving on the bank near the water's edge) may be pursued by males even here, though a capture on land was not observed.

The beginning of the attempt of a male upon a female is of course not in any way different from his approach toward another male but when he actually touches or often only nears the female his actions are usually very different for instead of the vigor and aggressiveness of the assailant rapidly falling off, as in case of one male approaching another, the aggressiveness is tremendously increased. The male makes every effort to catch the female if she is about to escape and often follows her under the water a little, but more often rapidly follows any disturbance of the surface and attempts to seize the female at once on her reappearance. If a male once succeeds in clutching with the fore legs any portion of the body of a female he cannot be dislodged, though the female continues to struggle. The male quickly brings himself to the pairing position, sitting upon the female's back. The fore legs are tightly clamped about the female with the first toes extended and deeply depressed against the ventral body wall of the female just back of the pectoral girdle. Often two or more males pursue a female at the same time and in case one male gets a hold on the dorsal side of the female and anterior to a second male the latter is most certain to be dislodged by the vigorous kicks of the other. If however a second male, as rarely happens, succeeds in getting well beneath the first so as not to be readily reached with the latter's hind legs the first male is very likely to be pushed upward and away from the female's body except for the clasping fore legs. In such a case he soon releases his hold. Amid the great preponderance of males the most vigorous and active ones are more likely to secure mates and to keep possession of them and effect fertilization of the eggs.

The female when in copulo does not so generally remain beneath the water as when unpaired, though much the greater part of the time is still spent upon the bottom or on submerged brush. It is possible of course that when a certain physiological state supervenes the female remains more at the surface than previously and this subjects her to the attacks of the males so that she does not long escape a mate, and that the pairing female does not remain more at the surface than she would if no male found her. It is certain that whereas the unpaired females are not rarely seen momentarily at the surface they very rarely remain for even a few seconds. The female is generally considerably larger than the male and is able to swim about readily with the smaller male upon the back. The male sometimes aids in swimming but usually remains passive regardless of the movements of the female. When approached by another male however the pairing male makes a vigorous defence with the hind legs and this with the struggles of the female usually serves to dislodge the intruder. But the struggles of the female and the resistance of the male are not always successful in warding off a second male. In the height of the pairing season there is usually to be seen one or more cases of more than one male clasping hold of a female. Such a multiple copulation is fraught with danger to the female as well as to the more successful males. There is a constantly recurring struggle on the part of the rival males for possession of the female. Unless the female is able to leave and remain beneath the surface these struggles are certain to attract other males which also attempt to get possession of the female. The result is a struggling, writhing mass of males holding on to the female and to the males already clasping the female. Each male strives to get into a more favorable position and (incidentally) to push off the other males. In one mass the female was lying on one side with the head under water and was apparently dead, while five males were holding to her and to one another in various positions and several other males were making occasional efforts to fasten hold on the bunch. Another such mass was lifted out in a dip net and when separated was found to consist of six males and the female. Five other males had been attempting to take part in the struggle but were not holding fast to the mass, avoided the net, and escaped. Sometimes the pairing female leaves the water but since she does not stray far from the water's edge she is frequently followed by one or more males.

Copulation may continue for a day or two. Pairs were kept in the laboratory together for thirty-six hours or longer. The embrace is so strong that in forcibly dislodging the male one fears lest the force employed must break the animal's leg. One male was found clasping a small male green frog and another late in the breeding season was found clutching a dead and somewhat decomposed female wood frog.

The wood frog first appears at the ponds in spring after a general thaw and several successive warm days. So far as observed at the Cut-off Pond the mating season was from March 30 to April 4 in 1911; March 28 to April 5 in 1912; and March 15 to 24 in 1913. It is possible that these do not represent the extreme dates for the end of the season. In one other pond near Cold Spring Harbor these frogs sometimes begin mating a day or two before they do in the Cut-off Pond and in a third pond wood frogs do not appear for several days after the first eggs are laid in the Cut-off Pond. Similar and much larger differences in times of egg-laying, in these three ponds, are observed with *Ambystoma punctatum*. These are presumably due entirely to temperature differences at the different ponds. One of these ponds is in a more sunny situation than the Cut-off Pond and the other is on a north slope.

The males apparently reach the pond first. In 1912 the chorus was in full activity March 28. The pond contained little water and there were perhaps 250 males in an area some 50 by 16 feet.

But no females were in evidence while on the following day they were as abundant as they ever appear. The females are much less numerous than the males. One is convinced that there are at least a dozen and possibly twenty males to every female. It would be easy to overlook the females entirely except during the height of the pairing season when by careful observation one can usually locate a few pairs, though the writer has never been able to find as many as a dozen pairs at a time in the whole pond, including those at the surface and the ones visible under water. The arrival at the pond was observed in the case of two females (forenoon of March 29, 1912). The behavior of one of these was noted as follows:

"Saw a female, a very light reddish orange one, enter the pond from the up-hill side. Saw her distinctly as she left the bank, made four or five bounds before reaching the water and plunged in without hesitation. Swam under at once and went for a distance of ten feet under the water, then came up again but dodged under when a male approached and went under a submerged leaf. She came to the surface again in seven minutes, then went under a leaf again and in the next five minutes repeated these movements two or three times. All the time she was getting farther away from the center of the chorus where she had entered the pond. Later she swam along the edge under water and away from the chorus and came to the surface twice, then swam across the pond under water and did not come up at once. Her actions could not be followed longer."

From such observations it seems evident that the females (and doubtless the later arrivals among the males) are attracted to the pond by the croaking males but that once in the pond the females endeavor to avoid the males, at least for a time.

The frogs generally remain at the pond only as long as the mating activities last. The females are apparently much weakened by the mating and egg-laying activities. Spent females were observed leaving the pond while the chorusing of the males was still at its height. Such females appear to be as attractive to the males as before laying the eggs and attempts to leave the pond are often frustrated by the active males.

The departure of males was less often observed and appears to occur in general only when the egg-laying is nearing completion, though a few males were seen well up the bank and apparently leaving the pond soon after the chorus had passed the climax of its activity. The sperm ducts of some of these were examined to see if perchance they might appear to be males which had discharged their sexual products, but no consistent difference was noted between those males leaving and the actively chorusing males still on the pond.

Most of the egg-laying, if the weather continues mild, occurs within two days. The eggs are laid in enormous aggregations, the bunches from the different females being crowded closely together. In 1912 all the eggs in the Cut-off Pond were laid within a radius of three or four feet. In 1911, and again in 1913, there were two such aggregations in the pond.

The indiscriminate trying by the males of every individual encountered aroused the writer's interest in the mode of sex-recognition. In an attempt to find the basis for sex-recognition a number of experiments were tried, in each of which a frog was fastened upon a light fishing line by hooking through the jaw and used as a decoy among the chorusing males at the pond. A long pole was used to hold and manipulate the decoy. The live frogs so used, unless tired out, swam about and acted apparently in a normal fashion, though the active females could not be kept at the surface without considerable manipulation.

The following notes were made on the spot, most of them March 30, 1912, and illustrate characteristic behavior of the mating frogs.

Experiment 1.—A male which had been pairing with a female in the laboratory all night was forcibly separated from its mate and tried on the line but with no further result than to be approached or tackled by practically every male which happened near him.

Experiment 2.—Placed a male paralyzed in the hind legs on the line and kept it moving somewhat. While tackled many times it was quickly released and in fact more often was merely quickly approached and not actually touched. One male however took hold and clasped him for several seconds, perhaps forty, but released him readily when I tried to simulate movement of a female by moving the line to which it was attached.

Experiment 5.—11 A.M., captured a female which had already deposited the eggs and was leaving the pond. This was used as a decoy and while one male approached somewhat and failed to grasp her the second and third made desperate efforts to get her and the latter succeeded in spite of her diving. The male dived after her but seemed unable to follow her well under water and caught her when she came near the surface and was soon in normal position.

Experiment 8.—Separated a pair and placed the female (still with eggs) on the line. She was soon captured, though she appeared to make every effort to dodge and escape the males.

Experiment 9.—A female, a large reddish brown one, was removed from a pair and placed on the line. There was seldom a male lost opportunity to grasp her and two or three would pursue her at once and fight vigorously for possession. Often I pulled her out of the water with a male ventrad and one dorsad though the former always let go before being swung in. Caught as many males as I wished by simply allowing them to grasp her and then swinging her in shore. Hauled in five in this way in less than three minutes and nearly all that time was spent in freeing the female for she was grasped as soon as brought near a male whether she was dorsal or ventral side up, at the surface or almost submerged. An hour later she was killed (pithed) and males seized her apparently quite as quickly and vigorously and struggled quite as hard for possession as before. Later when stripped of her eggs she was seized almost, though I think not quite, as readily as before. But it was getting late in the day and the air was becoming considerably cooler.

Experiment 10.—A dark female captured while leaving the pond on the up-hill side was used as a decoy. This one was so like a male in color, size and general appearances that it was only by examining the first fore toes and opening her that I could be positive of her sex, though I suspected it by the behavior of the males. It would seem that the frogs could scarcely recognize her as a female by sight. While she was not tackled as often or as vigorously as the reddish brown female (Exp. 9) yet during the hour she was observed she was paired with by five different males, none of which released her except when forcibly removed.

Experiment 14.—A dark female, *with eggs*, was paired with readily though my impression was that it was not so eagerly pursued and seized as the reddish brown ones.

Experiment 19.—Thinking a chemical sense perhaps involved in sex-recognition, tried a male with the contents of the cloaca and uterus of a female smeared over the posterior portion of his body. There were no more reactions than common to a male though I tried several times and with the cloacal parts of two females, one of which was the reddish brown one (Exp. 9) which had seemed particularly attractive to the males and with which so many males had been captured. The male probably did not retain the material on his body long when moved in the water. Several times however the decoy was gotten near a male before there had been much chance for the material to become washed off.

From these and other experiments it was made clear that the females alive or dead with or without eggs were recognized by the males though the dark ones without eggs were not so eagerly seized as the reddish brown ones and the dark ones still with the eggs were probably less readily recognized than the brown ones.

As regards sex-recognition the behavior of these frogs may be stated as follows. The males test every frog or moving object within a radius of several feet. As compared with the male the different behavior of the female in the pond probably serves as a partial means of sex-recognition. One gains the impression that he can distinguish a female in the pond as far as she is readily visible, for the female swims with long gliding strokes mostly under water, usually comes to the surface only momentarily and dodges under on the approach of another frog; while the males swim at the surface, swim about with short ineffective strokes and commonly make little effort to avoid an approaching frog. On the other hand occasionally a female swims at the surface with movements apparently indistinguishable from those of a male and on at least two occasions such females were pursued with remarkable persistence by one or more males. It is to be noted in this connection also that a rapidly swimming or persistently swimming male or the struggles of more than one male over a female attract males from all directions. Hence sex-recognition on the basis of method of movement can be only very tentative and preliminary.

The female wood frog when attacked by a male apparently makes every effort to escape both on the approach of the male and when he attempts to gain a hold on her. After the hold is once gained her struggles cease. When the male is approached by another male he sometimes apparently resists as vigorously or perhaps even with greater effect than the female, for she is heavy with eggs. But more often the male makes little or no show of resistance and is apparently as quickly left alone whether he resists or not. Hence there seems no consistent difference in the resistance offered by the two sexes when seized by a male.

The females at the breeding season, at least before the eggs are laid, are nearly all in the reddish brown color condition while the males are much darker. But exceptions to this color distinction between the sexes have already been noted. It is possible however that the color of the female plays some part in sex-recognition, for when used as decoys the dark females which had deposited the eggs were less frequently paired with than the reddish brown ones and this was possibly true to some extent with a dark female which still retained the eggs.

Thus it appears that the color of the female may *possibly* be a factor and that the peculiar behavior of the female is *probably* a factor in sex-recognition. But there seems unquestionably another factor involved when the male approaches closely or touches his prospective mate. Males were seen time and again to approach eagerly to within a few inches of other males only to turn back without coming into contact with or actually attempting to seize them. To be sure two or three instances of such behavior were noted when a male approached a female but they were most exceptional. When a male approaches nearly to a female his activity increases tremendously, in many cases before he can have actually touched her. On one occasion a male was seen to stop swimming within eight or ten inches of an unpaired female which was resting quietly at the surface. After a short time, perhaps ten or twenty seconds, with a rapid and vigorous movement he suddenly seized the female. The suddenness and rapidity of the movements suggested to the observer that possibly sex-recognition may have become complete at this distance before the male moved. No similarly vigorous attack upon a quiet male was observed though at times part of the pond contained a male frog for almost every square foot of surface.

Dead females are distinguished from dead males.

The readiness with which the attempt of a male to pair with another male is given up on near approach, the keenness of the male's pursuit after once approaching very near or touching a female, and the discrimination between a dead male and a dead female particularly in cases in which, to the human eye, the latter is indistinguishable in size, color and general features from a male, suggest that a chemical sense in involved in final sex-recognition though one experiment designed to test this hypothesis was unsuccessful.

The writer regrets his inability to further pursue the subject experimentally, but the pressure of other work left no opportunity to work on the problem during the height of the breeding season in 1913 and such will probably be the case in future seasons.

OBSERVATIONS ON BLOW FLIES; DURATION OF THE PREPUPAL STAGE AND COLOR DETERMINATION.

PHINEAS W. WHITING.[1]

The results of two main lines of experiment upon blow flies are recorded in the following paper. The first was concerned with the duration of the prepupal or migration stage of the larvæ and the conclusions may be summarized as follows:

The length of the prepupal period is determined by environmental rather than by hereditary factors and these factors are both complex and obscure. In general, dryness, cold, or agitation due to crowding, tend to prevent pupation, while change from dryness to dampness or the reverse, induces pupation. The prepupal stage may be extended for a long period, four months in one experiment (1912-*f*), in warm temperature without injury to the development of adult flies, which emerge from the pupæ in normal condition. Lack of opportunity for the larvæ to bury themselves does not inhibit pupation. Exhaustion of the food supply before the larvæ have attained full size has a tendency to produce undersized but normally formed flies. The causes producing misshapen and imperfectly expanded flies are more obscure, but may be in part due to drying of the pupæ. Delayed pupation in *Lucilia* larvæ is evidenced by a change from white to pink in the fat bodies, but in two genera of larger flies, *Cynomyia* and *Calliphora*, the white color is maintained although considerable shrinkage of the whole body occurs. There is no evidence that overfeeding delays pupation, but much evidence that larvæ will pupate immediately despite the fact that they have had abundant opportunity to overeat.[2]

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[1] Contributions from the Entomological Laboratory of the Bussey Institution, Harvard University. No. 76.

[2] From his studies on blow flies, Herms (Herms, Wm. B., '07, "An Ecological and Experimental Study of Sarcophagidæ with Relation to Lake Beach Debris," *Jour. Exp. Zool.*, IV., 1) reaches the conclusion that an optimum of development is attained by the larvæ after a certain period of feeding and that continued feeding after this has a tendency to delay pupation. In many of my cultures, however,

The second main line of investigation was concerned with coloration in the adult flies and showed results as follows:

The first color assumed by the adults after their integument has hardened is a deep purple which rapidly changes to dark metallic blue in the larger forms experimented upon, *Cynomyia* and *Calliphora*, becoming greenish after a short time in the males of *Cynomyia*. In *Lucilia* the purple passes rapidly into a bright green, which is later replaced by more or less bronze. The degree of this bronzing tendency is evidently of an hereditary nature as different strains vary in this respect and selection is here effective. The environmental factors, light and temperature, seem to have no effect upon the degree or rapidity of this process.

The experiments recorded in this paper were performed upon the various species of blow-flies common in New England, especial attention being given to the common green-bottle fly, *Lucilia sericata*.[1]

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larvæ of *Lucilia sericata* and *Cynomyia cadaverina*, which have been allowed to feed on all the fish that they would eat, on attaining the migration stage, have pupated within two or three days and have emerged from the pupæ in due course despite the fact that they have had plenty of opportunity to overeat.

In Table IV., Series 1, Herms records the mean weight of larvæ of *Lucilia* at the end of a feeding period of 60–72 hours as 38.183 milligrams, from 256 counts. This weight is regarded as the optimum for development. Among this series were a number that delayed pupation. These comprise Series 7. When weighed (there were 64 individuals) the mean weight was 33.59 milligrams. Although this weight is lower than the mean of Series 1, *it is considered*, that at the time of migration it must have been higher and that the difference is due to loss of moisture during the extended migration period, which had up to the time of weighing been twelve days. At the end of twenty days in the larval period as against a normal of about six days, 39 of these larvæ pupated, the rest being dead. *It is assumed* "that these larvæ were beyond the optimum weight, and for this reason pupation was deferred."

[1] Experiments were performed upon *Lucilia sericata* Meig., *L. sylvarum* Meig., *L. cæsar* L., *Calliphora erythrocephala* Meig., *C. viridescens* Desv., *C. vomitoria* L., and *Cynomyia cadaverina* Desv. Nothing has been done with the Sarcophagidæ, s. str., the flies named being included in the Calliphorinæ of the Muscidæ. Herms groups them all under the Sarcophagidæ, stating that he follows Girschner in this matter; but Girschner includes Calliphorinæ as his second group and Sarcophaga, Dexia, etc., as his fourth group under the family Tachinidæ, dropping the name Sarcophagidæ. (Girschner, E., '93, "Beitrag zur Systematik der Musciden," *Berl. Ent. Zeits.*, XXXVIII., 3).

While the work of Herms is said to have been done on *L. cæsar*, this is probably not correct as this species is relatively infrequent as compared with *L. sericata*, and Herms's material was collected from fish put out where all flies had ready

I have frequently found in my cultures imperfectly formed pupæ, sometimes misshapen and sometimes of normal form but with a soft covering. These have given rise in a few cases to undersized but normally formed flies, which lived as long as the full-sized individuals. In the majority of instances, however, the flies emerging were of normal size insofar as the chitinous parts of the body were concerned, but the abdomens appeared shrunken, the wings expanded only imperfectly, and the pigmentation failed to take on its customary brilliancy, remaining dull and opaque. This type of fly has also frequently appeared from pupæ apparently normal. In the case of *Luciliæ* which vary from a brilliant metallic green or greenish blue to a bright copper color becoming duller with age, the imperfectly formed flies resembled senescent individuals, being a dull coppery red. In the other larger species which are normally dark metallic blue with pollen that varies in amount in the different species and in the different individuals of the same species, the imperfectly expanded flies were dark blue in color without polish. That these imperfectly formed flies were hindered from normal development by drying of the pupæ, while small flies are produced from underfed larvæ might seem a reasonable explanation were it not

access, no distinction being made between the species of *Lucilia*. The relative abundance of *L. sericata* in the vicinity of Boston is especially to be observed where flies are crowded about a food supply in large numbers in which case it is only rarely that *L. cæsar* is taken. I have collected thousands of flies in the vicinity of the garbage scow, Boston, and at meat near the Bussey Institution, Forest Hills, and have found that less than one per cent. have been *L. cæsar*, while *L. sylvarum* has never been taken in such situations. At a very short distance from the Bussey Institution, however, where flies are relatively much fewer, *L. cæsar* and *L. sylvarum* have equalled and even surpassed *L. sericata* in numbers. I have never taken them, however, in any abundance. It is possible that *L. cæsar* may be more abundant in the region of the Great Lakes where Herms's work was done.

Calliphora erythrocephala is common about Boston all through the summer months while *viridescens* and *vomitoria* are rarely seen except in the spring and fall. *Cynomyia cadaverina* occurs only in the spring and fall and seems entirely to disappear during the summer. That these variations in frequency are entirely due to temperature and that there is no necessary periodicity in the breeding habits of the flies is evidenced by the facts that the forms disappearing during the summer still continue to breed farther north and that any of the species may be bred throughout the year by the proper regulation of temperature conditions. *Luciliæ* and *Calliphoræ* have been bred throughout the winter at Cambridge by Dr. A. O. Gross and *Calliphoræ* and *Cynomyia* have been bred during the entire year at Forest Hills by myself.

for the fact that in many cases the full-sized imperfectly formed specimens have come from pupæ kept damp. Nor can this be due in all cases to drying of the larvæ in the prepupal stage as will appear from experiment 1912-*f* recorded below.

Rarely has there been any considerable prolongation of the pupal stage in my experiments. Eclosion has taken place in approximately the time expected varying slightly with the species and the temperature. In one case, however, a few pupæ of *L. sericata* were obtained which failed to emerge and were kept for a number of weeks in a warm room in damp sand. At the end of that period they were examined for the possible presence of parasites, but nothing of that nature was found. I have no explanation to offer for this unusual fact.

On August 8, 1912, a female specimen of *L. sericata*, 1912-*f*, was taken at the garbage scow, Boston. She laid a number of eggs in less than a week and the larvæ soon reached the migration stage. This occurred before August 20. They were then placed in dry sawdust at room temperature and shortly there appeared thirteen flies, four males and nine females. None of the other larvæ pupated and at various times they were removed and examined. They seemed to be in a normal condition and reacted negatively to light. It was observed that the general tint of the larvæ gradually changed from white to light pink due possibly to the exhaustion of nutriment in the fat bodies. On October 23 they were all very pink but were still active although they had been in dry sawdust at room temperature for at least two months. At this time they were placed in damp sand and pupæ were soon formed from which thirteen males and eleven females emerged from November 11 to November 19. On November 20 and 21, several more flies emerged but unfortunately these were not counted. By December 4 the sand had dried somewhat and an examination showed nine larvæ remaining. The sand was dampened and on December 24, two flies emerged; on December 31, two more; and on January 3, one fly came out. This was the last of the lot, the other four having been killed on December 25 and studied for the presence of parasites, such as bacteria, etc. Nothing of this sort was found, however.

We have here then four larvæ which survived in the prepupal stage for at least four months at room temperature. The experiment indicates that in this case dryness has been the cause of the delay.

From the pupæ formed by these larvæ, there emerged after the usual time, flies which were of full size and completely expanded. The only difference observed in them from flies having a short prepupal period was the fact that the abdomen was contracted in a dorso-ventral direction. This deficiency, however, was corrected after a few days of feeding and the flies lived for a normal period of time. An extended prepupal stage under warm and dry conditions does not then necessarily produce misshapen flies, which often appeared from pupæ formed directly after the feeding period of the larvæ and which were permanently deformed, having the abdomen distorted and shrunken laterally as well.

From this experiment I conclude that hibernation may be undergone in the prepupal period. It is my opinion also that the majority of hibernating flies pass the winter in this condition and that the moistening of the soil in the spring by the melting of snow and the rain induces them to pupate. Many of the flies appearing on warm days in winter probably come from pupæ which are apparently not hindered from eclosion except by extreme cold.

That drying is not the only cause for delayed pupation, I am led to believe from the fact that in many instances larvæ have refused to pupate when buried in sand of all degrees of moisture in my various cultures both of *L. sericata* and of *C. cadaverina*. While this prolongation of the larval stage was in some cases undoubtedly due to cold, this cause could not be assigned to all cases as they often refused to pupate in summer temperature in moist sand.

A female of *L. sericata*, 1912-*V*, was taken at the Bussey Institution on November 20, 1912. One lot of her larvæ comprising 151 individuals reached the migration stage on December 4 and 5. They were distributed in eleven glass jars, but since conditions were the same in some of the jars I have grouped them for convenience into five series which represent the five conditions offered. They were kept at room temperature in semidarkness during the day and complete darkness at night. Without exception the flies emerged from the pupa cases in due time. Table I. gives the record of this experiment.

TABLE I.

RECORD OF FIRST LOT OF PROGENY FROM *L. sericata* 1912–*V*.

Series No.	Material Placed in Jar.	No. of Larvæ Placed in Jar Dec. 5, 1912.	No. of Pupæ Formed.				
			Dec. 8.	Dec. 12.	Dec. 16.	Dec. 24.	Jan. 3.
1	Dry sand.	45	22	4	5	12	1 (1 larva dead)
2	Damp sand	45	38 (2 larvæ escaped)	3	1	1	
3	Damp loam	30	13 (loam somewhat dry)	1 (loam dry)	0 (loam dampened)	2	9 (5 larvæ left)
4	Damp sawdust	15	12	(3 larvæ escaped).			
5	Damp cloth	16	4	10 (1 larva dead)	1		

In the first place it is obvious from this table that drying has acted as an inhibitor to pupation while moisture has accelerated it. Thus in Series 1 we notice considerable delay in pupation produced apparently by the dryness of the environment. In Series 2 on the other hand, the damp sand furnished a very favorable condition for pupation. In Series 3 are a few pupations at first, and then with the drying out of the loam, pupation ceases. Moistening the medium again produces pupation. The very favorable conditions in Series 4 and 5 may be due to the fact that the sawdust and cloth were kept very moist. Series 5 shows also that it is not necessary that the larvæ should have a chance to bury themselves in order to pupate, and this I have observed in large numbers of cases in my cultures where the larvæ have readily pupated in wooden boxes as soon as they have left the fish. There is one other thing noticeable here which was not observed in the first experiment and that is that the larvæ in the dry sand although slightly slower in pupating than the others have not entered a condition of indefinite prolongation of the prepupal period as in the case of 1912-*f*. My only explanation for this is that the temperature being higher in August than in November, drying out had been more rapid at the former time and thus a condition was reached in which the larvæ had not enough moisture to be able to pupate. This was supplied them in the later steps of the experiment.

The following experiment brings out another disturbing factor which acts as an inhibitor to pupation.

One hundred and seventy-six full-grown larvæ were obtained on December 11, 1912, which were also progeny from the later layings of the same *L. sericata* ♀, 1912-*V*. These were put into very damp sand crowded in a glass jar and kept at room temperature. On December 27 it was found that only 36 had pupated. The remaining 140 were distributed in five jars and their subsequent history is recorded in Table II.

TABLE II.

RECORD OF SECOND LOT OF PROGENY FROM *L. sericata*, 1912–*V*.

Series No.	Material Placed in Jar.	No. of Larvæ Placed in Jar Dec. 27, 1912.	No. of Pupæ Formed.				
			Jan. 3.	Jan. 19.	Feb. 5.	Feb. 15.	Mar. 14.
6	Dry sand	30	29	1			
7	Damp sand	30	7	0 (sand dry but dampened again slightly)	6 (sand dry and left so)	2 (19 larvæ including those from series 8 transferred to damp cloth)	19 dead flies found in bottle
8	Very damp sand	30	20	4	2 (sand dry, remaining larvæ put with Series 7)		
9	Dry bottle	30	30				
10	Damp cloth	20	7 (cloth dry and left so).	8	5		

From the delay in pupation from December 11 to December 27 it would appear that the crowding tended to inhibit pupation.

The larvæ were packed in so closely that their movements could be felt by each other and for this reason they were kept active. Upon being separated into smaller lots they soon pupated with the exception of Series 7. For the delay in this case I have no explanation, and it would appear that there are other unknown disturbing factors.

A female specimen of *Calliphora viridescens* (1912-21) was taken in the vicinity of the Bussey Institution, November 20, 1912. Eggs were soon deposited and the larvæ attained full growth on or before December 11. On that date they were placed in dry sand. No pupæ were formed and the larvæ were transferred to damp sand on December 18. Another transfer was made after about one week as pupation had not appeared and the larvæ were placed in dry sand. On January 7 a count was made and there were found to be 3 pupæ, 271 living larvæ, undersized and wrinkled, and 13 dead larvæ, wrinkled and dry. Of the three pupæ, 2 failed to emerge, although flies were formed inside, and the other emerged normally. The larvæ were placed in a jar with very damp cloth. Examination was not again made until January 30 when 2 normal flies were found, 1 ♂ and 1 ♀, 20 dead larvæ, 91 pupæ, and 158 living larvæ. The cloth was still damp. The larvæ were then transferred to a dry glass jar, and by February 15 were all dead and dried up, except that nine misshapen pupæ were formed which did not emerge. Of the other 91 pupæ which were kept on damp cloth only twelve had emerged by February 15, giving six males and six females. The others were dead.

The ill luck in getting these larvæ to pupate may be explained, I think, by the fact that they were at first crowded and thus disturbed each other. This was the condition up to January 7, a period of at least twenty-seven days, when they were observed to be undersized and wrinkled and some of them had died. Apparently in this case we have a condition very different from that of the first experiment recorded in this paper. It is possible that this may be due to the generic difference of the flies as prolongation of the larval stage of *Luciliæ* has often been observed to produce the pinkish coloration of the fat bodies, while in all cases the larger species, *Calliphoræ* and *Cynomyia*, have retained their white color but have become more contracted. In general the larger species pupate more readily than the *Luciliæ.*

In no case has there been any correlation of abnormalities of chætotaxy either with imperfectly formed flies, or with perfectly formed undersized individuals. My counts include several thousand specimens of various sizes and the lack of any correlation between number of bristles and size has been so obvious that I have made no measurements to establish this principle. I can, however, furnish numerous specimens of full size with greatly reduced chætotaxy and numerous minute specimens having the full number of bristles and even additional ones. Walton,[1] however, from a count of ten specimens of *Belvosia bifasciata* Fabr. concludes that the larger specimens have additional bristles while the smaller individuals are likely to show reduction. As I have not made a study of the parasitic Tachinid flies, with respect to chætotaxy I am unable to pronounce upon the correctness of this conclusion. Number of bristles appears to be an hereditary matter in the blow-flies, Calliphorinæ,[2] and as yet there is no sufficient evidence that environmental factors enter into their determination.

The normal color changes of the fly after eclosion are of interest and may well be described here for comparison with the abnormal conditions. In all the species bred, the fly emerges from the puparium by pushing off the cap by means of the ptilinum. The insect is at first very small and shrunken, but in a few minutes the ptilinum is withdrawn and the tracheæ filled with air. Thus the fly immediately assumes a size much larger than the puparium from which it has just emerged. The color is now white with pinkish and bluish tints, which deepen in a few minutes until at the end of one half hour after eclosion they become deep purple. During the next hour this changes to the normal metallic blue in *Calliphoræ* and *Cynomyia*, the males of the latter genus gradually taking on more or less of a dark greenish color in the course of a few hours. In *Luciliæ* the condition is very different inasmuch as the deep purple gives place to bright metallic green which is the color most in evidence at the end of one and one half hours after eclosion. If the fly be anæsthetized with ether the change of pigment is inhibited and the deep purple color may be made to persist throughout life. This does not seem in any way to interfere with the normal activities of the insect.

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[1] Walton, W. R., '13, "The Variation of Structural Characters Used in the Classification of Some Muscoidean Flies," *Proc. Ent. Soc. Wash.*, XV., 1, Apr., 1913.

[2] Whiting, P. W., '13, "Observations on the Chætotaxy of Calliphorinæ," *Ann. Ent. Soc. of America*, VI., 2.

The assumption of the bronze color in *Lucilia sericata* was made the subject of some investigation and it was found that the factors governing the rapidity of production of this hue were to a great extent of an hereditary nature. Considerable variation occurs among the individuals in regard to the rapidity with which this change from green to bronze occurs and in general it may be said that this takes place in the males more rapidly than in the females. In both sexes, however, the bronze may appear in certain regions of the body before the purple has been replaced by green in other parts and it would appear as if in some cases the bronze followed the purple directly without the intervention of the green. Variation is also considerable as regards the position of the bronzing, in some cases the abdomen becoming bronze while the thorax is yet green, while in other cases the reverse occurs. No evidence is yet found for an environmental cause influencing the rapidity of bronzing, but the process appears to be altogether independent of light and temperature. As regards the latter factor, however, it is desirable that more thorough experiment should be performed under more perfectly controlled conditions. That the chief cause for bronzing, however, is hereditary appears from the following experiment.

A female of *L. sericata*, 1913-*E*, taken near the Bussey Institution, March 19, 1913, produced 39 males and 43 females. Of this lot those that reddened most quickly were selected and a mating was obtained from one pair. This pair produced 70 males and 77 females. A further selection was made from the reddest of these flies, which were examined in less than twenty-four hours after eclosion. One of the pairs selected produced a large family consisting of 366 males and 343 females. At this point the color selection was abandoned, the family being continued as a selection for additional bristles. A rapid effect of selection was noticed through the course of the experiment so that each succeeding generation averaged much more bronzy in appearance than the preceding. At the close of the experiment in the F_3 generation, properly the second generation of selection, practically all the flies assumed considerable of the bronzy color before they were examined, which was done once every day.

In the selection in the opposite direction the results were not as striking. A female of *L. sericata*, 1913-*F*, was taken near the Bussey Institution, March 19, 1913. She was chosen because she appeared greener than many of the others that were seen about the building. In a few days she deposited eggs from which 24 males and 19 females were reared. Many of these were bronze-colored as soon as they had hardened but a few were green after a period of two or three days with but a slight amount of bronze. A single mating obtained from a pair of these latter gave 93 males and 90 females which averaged much greener than the second generation of the red selection 1912-*E*. At this point the experiment was cut short by the death of the flies selected.

The flies of both the red and the green selections were placed in boxes and allowed to become dry. It was intended to group them into classes according to color and thus to demonstrate more clearly the hereditary nature of the bronzing factors. After the specimens had dried, however, it was observed that the pigment had changed considerably, the reds becoming much greener and the greens often being streaked with blue. It was therefore found necessary to abandon this more accurate proof of the hereditary nature of the bronzing until such time as another selection could be made and the colors of the flies recorded as soon as killed by comparison with standard color charts.

Experiments are now under way which it is hoped will throw more light upon the conditions governing the life histories and habits of these flies.

My thanks are due to Mr. C. T. Brues for helpful criticism in the preparation of this paper and for many suggestions throughout the course of the work.

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BIOLOGICAL BULLETIN

NO CROSSING OVER IN THE MALE OF DROSOPHILA OF GENES IN THE SECOND AND THIRD PAIRS OF CHROMOSOMES.

T. H. MORGAN.

In a brief note in *Science* for November 22, 1912, it was pointed out that there was no "crossing over" in the male between the factors for black body color and vestigial wings, while between the same factors in the female there was 22 per cent. of crossing over. The data then published have been extended and revised,[1] and the results can now be given in detail. For purposes of easy comparison I shall present the data in the same order as those given in the preliminary report.

It had been shown by Morgan and Lynch (Biol. Bull., XXIII., 1912) that when black flies having normal (long) wings are mated to gray flies having vestigial wings, only three classes appear in F_2, viz.:

Gray Long.	Black Long.	Gray Vestigial.	Black Vestigial.
4569	2151	1626	0

It was pointed out later (*Science*, 1912) that the absence of the black vestigial class would be expected, if, in F_1, no interchange of factors occurs in one sex. The following analysis will make evident why, on the assumption that there is no crossing over in the gametogenesis of the F_1 male, there are no black vestigials expected in F_2.

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[1] We have learned better how to control the conditions of culture so that less disturbance results from differential viability. Many of the old data, although consistent within themselves, are not strictly comparable with the recent data obtained under better conditions. For the case of black and vestigial the data presented here differ from those reported (*Science*, 1912) in that new data have been substituted for some of the old data in the tests of the amount of crossing over in the female.

Black long ♀, $bV_g—bV_g$.
Gray vestigial ♂, $Bv_g—Bv_g$.

F₁ ♂ or ♀, bV_gBv_g.

Gametes of F₁ $\begin{cases} BV_g—bV_g—Bv_g—bv_g & \text{Eggs} \\ bV_g—Bv_g & \text{Sperm.} \end{cases}$

F₂
BV_g bV_g, gray long, BV_g Bv_g, gray long.
bV_g bV_g, black long, bV_g Bv_g, gray long.
Bv_g bV_g, gray long, Bv_g Bv_g, gray vestigial.
bv_g bV_g, black long, bv_g Bv_g, gray vestigial.

The expectation is 4 gray long, 2 black long, 2 gray vestigial, 0 black vestigial. The data show that the gray vestigial run behind expectation, which is due to viability (crowding out through competition). It is evident that the expectation would be the same if the failure to cross over occurred in the female instead of in the male as here assumed. In order to test which sex failed to give crossing over the following matings were made. (1) Black, long winged females were mated to gray, vestigial winged males. The F₁ flies ♂ and ♀ were gray, long. The F₁ males were bred to black vestigial females and gave *two* classes.

Gray Long.	Black Long.	Gray Vestigial.	Black Vestigial.
0	19	19	0
0	30	33	0
0	34	20	0
0	193	115	0
0	174	118	0
0	542	416	0
0	992	721	0

If we assume that there has been no crossing over in the F₁ heterozygous males the result is explicable, as shown in the following analysis, except in so far as the gray vestigials are crowded out.

Black vestigial ♀, $bv_g—bv_g$,
F₁ ♂, $bV_g—Bv_g$,

F₂ bv_g bV_g, black long,
bv_g Bv_g, gray vestigial.

The F_1 heterozygous females (sisters to the males just tested) were also tested by crossing to black vestigial males. *Four* classes were produced.

Gray Long.	Black Long.	Gray Vestigial	Black Vestigial.
66	283	201	58
65	243	224	50
55	218	169	42
20	113	112	29
71	311	210	47
17	153	181	20
44	231	218	48
338	1,552	1,315	294

This result, in contrast to the last one, is explicable on the assumption that crossing over occurs in the female, as the following analysis shows:

F_1 ♀, BV_g—bV_g—Bv_g—bv_g,
Black vestigial ♂, bv_g— bv_g

BV_g bv_g, gray long.
bV_g bv_g, black long.
Bv_g bv_g, gray vestigial.
bv_g bv_g, black vestigial.

The expectation on the basis of *free* crossing over in the female is 2 gray long, 2 black long, 2 gray vestigial, 2 black vestigial. The two classes gray long and black vestigial are cross over classes, and their smaller numbers result from linkage. It will be noted that the two vestigial classes fall below the corresponding long classes, and this can be accounted for as due to viability. The percentage of crossing over on the basis of the data is 17.9.

These last two experiments show that the realized classes are explicable on the basis of non-crossing over of factors in the heterozygous male, and of crossing over to the extent of 17.9 per cent. in the female. This conclusion can be tested by bringing into the cross the same factors in other combinations. One such test is as follows. Gray, long winged females (BV_g) were bred to black, vestigial males (bv_g) and produced gray long males and females. The F_1 heterozygous males produced in this way were mated to black vestigial females, and gave:

Gray Long.	Black Long.	Gray Vestigial.	Black Vestigial.
18	0	0	18
90	0	0	95
174	0	0	78
102	0	0	86
384	0	0	277

This result is what is to be expected on the basis of no crossing over in the heterozygous male as the following analysis shows:

Black vestigial ♀, bv_g—bv_g
F₁ ♂, BV_g—bv_g

bv_g BV_g, gray long.
bv_g bv_g, black vestigial.

The F₁ heterozygous females (sisters to the males just tested) were in turn mated to black vestigial males and gave:

Gray Long.	Black Long.	Gray Vestigial.	Black Vestigial.
255	55	47	184
163	24	37	139
47	9	11	33
121	18	16	109
586	106	111	465

These results are explicable if crossing over (17.1 per cent.) takes place in the F₁ heterozygous females, as indicated below:

F₁, bV_g—BV_g—bv_g—Bv_g
Black vestigial, bv_g—bv_g

bV_g bv_g, black long.
BV_g bv_g, gray long.
bv_g bv_g, black vestigial.
Bv_g bv_g, gray vestigial.

It has been pointed out that when the factors for black and for long wings go in together, *i. e.*, enter with the same chromosome, and the factors for gray and for vestigial enter with the other chromosome (it does not matter which parent contributes each) the double recessive does not appear in F₂. In more general terms it may be said, for the second chromosome, that when two recessives enter, one from each side, the double recessive does not appear in the F_2 generation. The explanation of this is clear on an examination of the first analysis. Since there is no crossing over in the male, each gamete in the male will contain one or the other of the dominant factors, hence no double recessive zygote can appear, and every zygote must contain at least one dominant. This means that there is one class with two dominants, one with the one, one with the other. If we assume that the factors are carried by specific chromosomes, and that the factors for black and for vestigial (and hence for their normal allelomorphs) lie in the homologous chromosomes, we can explain the results just given on the basis that no interchange of factors occurs between these chromosomes in the male even though in the female interchange takes place. Why an interchange should take place in one sex and not in the other cannot be stated, but I should not be surprised if a thorough cytological study would throw some light on the subject.

The converse case is that of two recessives entering from the same side, in which case all the expected classes appear in F_2. An example with its analysis will make this clearer. Gray, long winged females (double dominants) were mated to black vestigial males (double recessives) and gave gray long winged F_1 offspring. These F_1 flies, mated, gave in F_2:

Gray Long.	Black Long.	Gray Vestigial.	Black Vestigial.
246	9	18	63

The following analysis shows why the result to be expected here is different from that in the last case. Half of the gametes

Gray long ♀, BV_g—BV_g.
Black vestigial ♂, bv_g—bv_g.

F_1 ♂ and ♀, BV_g—bv_g.

Gametes of F_1 bV_g—BV_g—bv_g—Bv_g Eggs
BV_g—bv_g Sperm

bV_g BV_g, gray long, bV_g bv_g, black long,
BV_g BV_g, gray long, BV_g bv_g, gray long,
bv_g BV_g, gray long, bv_g bv_g, black vestigial,
Bv_g BV_g, gray long, Bv_g bv_g, gray vestigial.

in the male contain both recessive factors and in consequence, even with no crossing over in the male, the realization of a double recessive zygote becomes possible.

The expected ratio without linkage in the female is 5 : 1 : 1 : 1. Four classes are expected and four appear, but the ratio given above is entirely changed by the linkage of the factors which here, on our assumption, lie in the same chromosome.

PURPLE EYES AND VESTIGIAL WINGS.

An eye color called purple also shows linkage with vestigial and is placed, therefore, in the second group of factors. It has likewise been used to test non-crossing over in the male. Purple vestigial flies mated to red-eyed, long-winged flies gave red-eyed, long-winged F_1 males and females. When these F_1 males were bred to purple vestigial females only two classes were produced:

Red Long.	Purple Long.	Red Vestigial.	Purple Vestigial.
52	0	0	62
141	0	0	113
96	0	0	131
28	0	0	34
68	0	0	89
22	0	0	33
112	0	0	90
519	0	0	552

The result is explicable if no crossing over occurs in the male and the analysis is the same in principle as that for the black vestigial case. As explained before, every sperm contains one or the other recessive since they went in separately and have not crossed over in the male.

When the F_1 females (sisters to the males just tested) were mated to purple vestigial males, the following results were obtained:

Red Long.	Purple Long.	Red Vestigial.	Purple Vestigial.
2,876	270	270	2,433

The result is explicable on the hypothesis of crossing over (about ten per cent.) in the female, as the analysis for the comparable case for black vestigial shows.

Purple-eyed, long-winged flies were mated to red-eyed, vestigial flies, and gave normal F_1 flies. When the F_1 males were bred to purple vestigial females the following results were obtained:

Red Long.	Purple Long.	Red Vestigial.	Purple Vestigial.
0	62	42	0
0	78	70	0
0	61	53	0
0	66	103	0
0	79	90	0
0	346	358	0

Here again the results are explicable on the hypothesis of non-crossing over in the male. The recessives enter the F_1 male from opposite sides, *i. e.*, they lie in different but homologous chromosomes. Hence if no crossing over occurs in the male each spermatozoon will contain one or the other recessive, and since the eggs are all alike and carry only double recessive factors only two types of zygotes are expected.

When the F_1 females, sisters to the males just tested, were likewise bred to purple vestigial males, the following classes result:

Red Long.	Purple Long.	Red Vestigial.	Purple Vestigial.
265	2,203	2,038	234

Crossing over in the female (about 10 per cent.) explains the classes that appear in this experiment.

These results with purple vestigial are comparable at every point with those for black vestigial and both furnish evidence of no crossing over in the male. The numbers are large enough to make it highly probable that crossing over in the male did not occur.

Further Examples of Non-crossing over in the Male Between Genes in the Second Pair of Chromosomes.

The following data are taken from experiments that Mr. C. B. Bridges and the author are carrying out. The records have been made by Mr. Bridges. A recessive mutant called "dachs" (from the short legs of the flies) was combined with another character also in the second group, viz., black. When the double recessive dachs black is crossed to the wild (normal gray) fly the offspring are normal. When an F_1 male was mated to dachs black females the following results were obtained:

Normal Gray.	Dachs Gray.	Normal Black.	Dachs Black.
98	0	0	72

These results are consistent with the hypothesis of no crossing over in the male. Since dachs lies on the other side of black from purple and vestigial, the data extend the application to a new section of the second chromosome, and considerably increase the significance of the results.

Another recessive mutant stock has wings curved in an arc. "Arc" was combined with black—both belonging to the second group of factors. When black arc was mated to wild flies (with straight wing) the offspring were normal. An F_1 male back crossed to black arc gave the following results:

Gray Straight.	Black Straight.	Gray Arc.	Black Arc.
161	0	0	145

This result also is conformable with the hypothesis of no crossing over in the male, and since arc lies on the same side of black as does purple and vestigial but considerably beyond either, still a new section is found to be free from crossing over in the male. Further, since in the female there is a very great amount of crossing over (about 36 per cent.) between black and arc, the lack of crossing over in the male is even more striking.

When black was crossed to purple the F_1 flies were gray red. An F_1 male was mated to a black purple female and gave in the next generation:

Gray Red.	Black Red.	Gray Purple.	Black Purple.
0	74	71	0

These results are again conformable with the hypothesis. In this case the two recessives entered from different sides.

Another mutant caaracter, streak, is a dominant. Streak gray mated to normal (thorax) black gave streak gray F_1 flies. An F_1 male was mated to the double recessive, normal black females, and gave the following classes:

Streak Gray.	Normal Gray.	Streak Black.	Normal Black.
19	0	0	21

Again the results conform. Here the two dominants entered from one side and two recessives from the other, and the double recessive normal black was used for testing the gametes of the F_1 male. Streak lies at the most extreme end of the chromosome so far as known, on the same side of black as does dachs, and on the other side from purple, vestigial and arc. The interval between streak and black is nearly as long as that between black and arc, so that the results are of interest because the whole section from streak on the one hand to arc on the other is known to give the same result, namely, a complete absence of crossing over. These two loci, streak and arc, are so far apart that in the female there is practically free Mendelian assortment.

No Crossing over in the Male between Genes in the Third Pair of Chromosomes.

A test of whether or not crossing over takes place in the male between genes in the third group; or, as we say, in the third chromosome, was first published for the case of pink and ebony by Sturtevant (*Science*, 1913), who showed that no crossing over occurred in the F_1 male for the small number of males tested. The following case gives similar results for another pair of loci. The data are taken from experiments that are being carried out by C. B. Bridges and the author.

A mutant stock has kidney-shaped eyes. A double recessive stock of pink kidney was made up, both factors being in the third chromosome. Pink kidney mated to wild flies gave F_1 flies with red round eyes. The F_1 males back crossed to pink kidney females gave the following results:

Red Round.	Pink Round.	Red Kidney.	Pink Kidney.
23	0	0	15
11	0	0	9
56	0	0	50
91	0	0	61
6	0	0	8
22	0	0	17
11	0	0	5
6	0	0	2
63	0	0	58
142	0	0	114
431	0	0	339

These results are in harmony with the hypothesis of no crossing over in the male between genes in the third group of chromosomes. The amount of crossing over between pink and kidney in the female is about 15 per cent., which is greater than the most frequent value of about 5 per cent. in Sturtevant's case of pink ebony.

Discussion.

In their paper of 1911, Bateson and Punnett described certain phenomena that they called coupling and repulsion. The numerical and class results offer many points of similarity to the cases here described. In a later paper they gave reasons for abandoning the earlier view of repulsion as distinct from coupling. In this paper (December, 1911) they gave two cases in which linkage occurs, but in which crossing over must be assumed to take place in both sexes. Bateson and Punnet postulate in order to account for their results that segregation takes place at some early stage in the germ-tract. Whatever form of interpretation may apply to the cases described by Bateson and Punnett, the tests that I have made of F_1 males and females show, that in *Drosophila* at least, the results are due to failure of crossing over between factors in one sex. It may be that in certain animals and plants crossing over is the same in both sexes, while in other cases it may be that crossing over is different in the two sexes. Whether this is true or not can only be determined by making tests like those here employed. Until such tests have been made for other forms in which linkage has been found we cannot know how widely the explanation here followed may be extended.

A NEW GENE IN THE SECOND CHROMOSOME OF DROSOPHILA AND SOME CONSIDERATIONS ON DIFFERENTIAL VIABILITY.

C. B. BRIDGES AND A. H. STURTEVANT.

Morgan and Lynch (Biol. Bull., '12) and Morgan (*Science*, '12) have reported the linkage relations of two non-sex-linked genes, black and vestigial.[1] Morgan considered these two genes as lying in a "second chromosome," the first chromosome being the sex chromosome. He showed that in the female there was a considerable amount of crossing over between these two genes, but in the male there was none at all so far as the data showed.

At the time when this linkage between black and vestigial was first observed we were engaged in a systematic search for linkage between non-sex-linked genes in *Drosophila*. One of us (Bridges) had already observed in the F_2 generation of a cross of black by curved[2] that no black curved flies appeared. We interpreted this case as one of linkage of such a strong order that no crossing over had taken place. On the basis of this linkage we concluded that curved was in the same chromosome as black, that is, in a "second chromosome." The similar work of Morgan on black and vestigial showed that the non-appearance of the double recessive in a case in which two second chromosome recessives entered the F_1 from opposite parents, could be explained on the basis of lack of crossing over in the male. The present paper shows that the same explanation applies to the case of black by curved, and further deals with the determination of the amount of crossing over in the female between the black and the curved loci.

An individual heterozygous for two allelomorphic pairs as *AB*, *ab* may form four kinds of gametes, namely *AB*, *ab*, *Ab*, and

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[1] Vestigial was at that time called wingless.

[2] "Curved" a wing mutant discovered by Bridges is characterized by the thin texture of the wings which are held out widely from the body and curved. This and other mutants are shortly to be described in detail by Morgan and Bridges.

aB. In case of linkage, however, the two new combinations (here *Ab* and *aB*) are not represented in as large numbers in the gametes as are the original combinations (*AB* and *ab*). The actual ratio in which these gametes are formed can be calculated only very indirectly from an F_2 zygotic ratio, and if the two F_1 individuals are forming the gametes in different ratios, the calculation may be impossible.

As suggested by Baur (*Verh. naturf. Ver. Brünn*, '11) what is needed is to test these double heterozygotes by an individual all of whose gametes are of one kind, namely, recessive for both factors in question (*aa*, *bb*) since in this case they will not mask any combination in the gametes tested. Baur's method, then, is to test double heterozygotes by double recessives. The proportion in which the zygotes appear is a direct measure of the gametic proportions. In such zygotic proportions there are two equal classes representing original combinations (in the example *AB* and *ab*) and two other equal classes representing recombinations or crossovers. In calculating the percentage of crossing over therefore, we add these cross over classes together and divide by the total number of zygotes. Since in the case of sex-linked characters the male producing sperm is analogous to a double recessive, the F_2 males will in effect always be such a back cross test.

Flies with curved wings from pure stocks were mated to stock black flies. The F_1 flies were gray not-curved, that is, like the wild fly in appearance. F_2 consisted of:

	Wild Type.	Black.	Curved.	Black Curved.
	391	194	168	0
	458	226	165	0
Total	849	420	333	0

The absence of black curved in F_2 is the result to be expected if there is no crossing over in the male, no matter what the percentage of crossing over may be in the female. However, if there is crossing over in the female a few of the F_2 blacks should be heterozygous for curved and a few of the curved heterozygous for black. The most advantageous procedure to get the double recessive from these F_2 flies is to mate in mass cultures the blacks of one sex to the curved of the other sex. In F_3 if there has been crossing over in the F_1 ♀ there should appear some of the double recessives or some single recessives heterozygous for the other single recessive. In fact some blacks appeared which gave, in F_4, 3 black : 1 black curved. From these black curved individuals a pure stock of the double recessive was obtained directly.

That the absence of black curved flies in F_2 was really due to lack of crossing over in the male was shown by making "back-cross" tests of doubly heterozygous males as follows:

Black × curved.
↓
F_1 ♂♂ × black curved ♀♀.
↓

Wild Type.	Black.	Curved.	Black Curved.
0	35	40	0
0	44	31	0
0	25	20	0
0	18	15	0
0	45	60	0
0	39	33	0
0	90	83	0
0	50	51	0
0	29	34	0
0	375	367	0

From the converse cross of wild by black curved described below, two tests of F_1 males were made, with the following results:

Wild × black curved.
↓
F_1 ♂♂ × black curved ♀♀.
↓

Wild Type.	Black.	Curved.	Black Curved.
108	0	0	88
71	0	0	57
179	0	0	145

No new combinations of characters (crossovers) appeared in the 1,066 flies from the two converse experiments. This is in exact agreement with Morgan's results on black vestigial, and is somewhat more significant, since, as will appear below, there is in our case more crossing over in the females than in the combination studied by Morgan.

We have seen that when black and curved enter F_1 separately, one class, namely, the double recessive, does not appear in F_2, for the reason that a double recessive gamete is not formed in the F_1 male through lack of crossing over. But if black and curved enter from the same parent then half the gametes of the F_1 male are doubly recessive, and, therefore, give in a back cross test the amount of crossing over in the female. The other half of the gametes of the male are the double dominant class and in consequence half of the flies fall into a single double dominant class. If, then, there is crossing over in the female in the ratio of one crossover gamete to n of the original combination, the F_2 will consist of $n : 1 : 1 : n$ zygotes from the doubly recessive sperm, and a like total, that is $2n + 2$, from the doubly dominant half of the sperm. The F_2 proportions expected are therefore $3n + 2 : 1 : 1 : n$ and the ratio of each single recessive class (1) to the double recessive class (n) gives the gametic ratio directly. The results of such a cross as that described appear below:

Wild × black curved.
↓
F_2

	Wild Type.	Black.	Curved.	Black Curved.
	298	27	15	48
	88	7	5	8
s*[1]	252	21	15	56
s*	310	19	28	79
	948	74	63	191

The occurrence of black and of curved flies in this experiment demonstrates that crossing over takes place, and from the evidence of the preceding experiments, we conclude that it must have been in the females. That this is the correct interpretation is shown by direct tests of such females. As stated above, the percentage of crossing over can be calculated directly from tests of this nature. In the following table the calculation for each culture and for the total is given.

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[1] In all counts reported here *s* signifies that the record includes only the offspring of a single female. For reasons which will appear in future publications from this laboratory it has seemed advisable to include only the first cultures obtained from any females. When later cultures are available but have been omitted, the record will be marked with an asterisk.

Wild × black curved
↓
F_1 ♀ × black curved ♂♂.
↓

	Wild Type.	Black.	Curved.	Black Curved.	Per Cent. of Crossovers.
	96	31	21	96	21.3
s*	63	20	17	78	20.7
s*	103	34	40	102	26.5
s*	106	27	44	105	25.2
s*	112	34	55	127	27.2
s	130	38	49	144	24.1
	610	184	226	652	24.5

Similar tests of F_1 females from the converse cross of black × curved gave:

	Wild Type.	Black.	Curved.	Black Curved.	Per Cent. of Crossovers.
	88	203	212	80	28.8
	68	161	159	80	31.6
	56	221	256	69	20.8
	67	373	286	84	18.7
	70	252	240	64	21.4
s	17	97	95	26	18.3
s	27	113	92	21	19.0
s	33	152	141	28	17.2
s	18	100	99	27	18.4
s	11	50	56	8	15.2
s*	45	144	150	55	25.4
s*	41	134	119	52	27.1
s*	56	157	148	40	23.9
s*	47	135	95	29	24.8
	644	2,292	2,148	663	22.7

Adding the figures from these two converse experiments gives 1,717 crossovers in 7,419 flies, or 23.1 per cent.. of crossovers. Because of double crossing over, which is known from unreported experiments to occur within this distance, 23.1 is slightly less than the actual chromosomal distance apart of these two loci.[1]

It will be seen from the two above tables that there is apparently a rather wide range of variability in the percentage of crossing over in different cultures. This variability is much greater than one would expect to find if it were due entirely to chance deviations; and one might therefore be led to suppose that it is due to an actual variation in the strength of linkage. While this conclusion may be correct, it is not necessary, since there is another important factor which must be considered—namely, the effects of differential viability. In order to get definite data regarding the manner of action of this disturbing element we have made some crosses in which it may be studied without the complication of linkage. If curved flies heterozygous for black ($B\ c_v\ b\ c_v$) be mated to blacks heterozygous for curved ($b\ C_v\ b\ c_v$), the same four classes of flies as in the above tables should be produced, but now in equal numbers. If equality is not shown the deviation cannot be due to linkage, but must probably be attributed either to the error of random sampling, or to differential viability. The results actually obtained in these experiments are shown in the following table:

---

[1] See Sturtevant (*Jour. Exp. Zool.*, '13) for a discussion of linear series of genes within a chromosome.

Black[1] (het. for c_v) × curved (het. for b).
↓

	Wild Type.	Black.	Curved.	Black Curved.
I.*s*......	31	31	22	29
II.......	46	48	40	48
III.......	35	27	30	14
IV.*s*......	44	53	33	65
V.*s*......	14	31	18	22
VI.......	52	50	56	41
VII.......	47	31	24	36
VIII.*s*......	43	31	2	20
IX.*s*......	13	7	10	15
X.*s*......	37	43	43	28
XI.*s*......	37	51	36	26
XII.*s*......	14	14	14	17
XIII.*s*......	76	78	56	46
	489	495	384	407

That there really is no linkage present is evident from the totals, since the complementary classes give totals of 879 and 896, respectively. The deviation of these numbers from equality is less than half the standard error—a very close approximation to expectation. These totals show some effects of differential viability, especially in that the curved flies were less numerous than those with normal wings. A study of the individual bottles brings out some other interesting points. It is obvious that viability may not always produce the same kind of effect. For instance, in culture VIII., the curved class ran far behind all three others, yet in VI. it was the largest class of all. The same relations are shown by the black curved class in XIII. and IV. respectively, and in several other cases. It also appears that the viability difference between two classes differing in two characters is not always merely a summation of the effects produced by these characters separately. Thus in culture VII., since the curved and the black class are each behind the normal class, we might expect the class which is both black and curved to be still further behind—yet it is really ahead of both single recessive classes. In culture VIII. black is slightly behind normal, but black curved is far ahead of curved (gray). It is obvious from these considerations that it is not possible to work out "coefficients of viability" and use them for making corrections in our data, since with respect to viability the deviations are not constant in amount or direction. However, it is to be noted that when conditions are made as favorable as possible the error from viability is reduced considerably, and often becomes very slight indeed. There is evidence which indicates that differential viability is often due to unequal sensitiveness to starvation, dryness, or similar unfavorable conditions. Several of the cultures recorded in the last table above were purposely kept under various poor conditions (small bottles, little food, etc.,) in order to test this point. Cultures II., III., and VIII. are examples showing the results produced, and III. and VIII. are among the most aberrant cultures in the table. The remedy, then, would seem to be in choosing mutants which are of nearly the same vigor as the normal, and in keeping the cultures in good condition—plenty of room and good food.

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[1] The first five cultures below were from curve ♀ × black ♂, the others from the reciprocal cross (black ♀ × curved ♂).

Even under these conditions there may be a high mortality, but that this need not always be a *differential* mortality is indicated by an experiment which we have carried out. Three females from the cross of black by curved were tested by backcrossing to black curved males. The eggs were counted daily, and the offspring produced were recorded, with the following result:

Wild Type.	Black.	Curved.	Black Curved.	Total.	Total No. Eggs.
34	91	96	32	253	550

Thus although less than half the eggs produced flies, there is no evidence of differential variability, since the complementary classes are approximately equal, as expected.

Columbia University,
January, 1914.

THE INFLUENCE OF THE ENVIRONMENT ON THE SIZE OF EXPECTED CLASSES.

T. H. MORGAN AND SABRA COLBY TICE.

In crosses in which rudimentary wings are involved, it has been apparent, since this race was first bred, that the classes containing rudimentary wings often run far behind expectation. The experiments made clear that the character rudimentary wings is a Mendelian recessive and is sex-linked. The deficiencies that appeared were assigned to viability of these flies. We have found meanwhile for other stocks that by breeding *pairs* of flies in large bottles, with an abundance of food, kept in good condition, there was a very marked increase in number of those classes that are deficient in number if many flies are bred in small bottles, or even in large bottles if so many parents are used that crowding of the larvae takes place. It was determined, therefore, to repeat the experiments with rudimentary wings under the most favorable conditions that our experience had made known to us. In order to avoid the possible criticism that the stock might have changed, a control culture *en masse* was again made in which crowding took place.

Results similar to these with rudimentary wings had also come up in crosses in which a new mutant, "strap wing" was involved. This factor is not sex linked, but belongs to our second group. Similar experiments were carried out with this stock.

THE VIABILITY OF THE RUDIMENTARY WINGED RACE.

In a paper in *Science*, 1911,[1] an F_2 count is given in which 5,850 long-winged flies (♂ and ♀) and 83 rudimentary-winged males are recorded. The expectation is that of these 5,850 flies one third should be long-winged males, or 1,950. This number is also the expectation for the rudimentary-winged males. Instead of 1,950 there are only 83 males or 1/23 the expected number.

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[1] *Science*, Vol. XXXIII., March 31, 1911.

The F_1 generation of the reciprocal cross was published in *Science*, 1912.[1] The rudimentary-winged female bred to a long-winged male gave 381 long-winged daughters and only 3 rudimentary-winged sons where equality was expected. Whether the lack of sons here is due entirely to viability, or to other conditions as well cannot be stated.

In a very brief paper in 1911[2] some other data were given that showed the rudimentary classes running behind expectation. These data were corrected and expanded in another paper,[3] to which reference may now be made. An F_2 count is given there, that is an extension of the data published in the first paper referred to above. There are 14,309 long-winged grandchildren (♂♂ and ♀♀) and 115 males with rudimentary wings. The expectation here is that one third of 14,309 or 4,769 flies should have rudimentary wings. The entire number is 115, or only 1/41 of the expected number.

There is an F_1 count of the reciprocal cross when 68 long-winged daughters and 3 rudimentary-winged sons appear. This ratio is not lower than that given above, and may safely be ascribed to viability. In the F_2 count of the same combination the following classes and numbers were realized:

Long ♀	721
Long ♂	698
Rud. ♀	163
Rud. ♂	237

The expectation is for equal numbers. The rudimentary males, while far behind expectation, are not so far behind as in other crosses cited, which is due to better treatment. This same statement applies to the remaining data of the 1912 paper for, at that time realizing more fully the influence of the environment on viability larger bottles with more food were used. Since most of the data involves miniature wings it is not cited here.

In the new experiments rudimentary-winged males were bred to long-winged (wild) females; and long-winged daughters and sons obtained. The daughters were then back-crossed to rudimentary males either in pairs or *en masse*. When bred *en masse* the following offspring were obtained. The expectation is for equality in all four classes:

---

[1] *Science*, Vol. XXXIV., March 22, 1912.

[2] *Proc. Soc. Exp. Biol. and Medicine*, VIII., February, 1911.

[3] *Zeit. f. induktive Abstammungs und Vererbunglehre*, VII., 1912.

Long ♀	527
Long ♂	489
Rudimentary ♀	7
Rudimentary ♂	31

There is an enormous deficit in the classes of rudimentary flies. Instead of equality there are only 1/27 as many as expected.

When on the other hand the F$_1$ females were back-crossed *in pairs* the following totals were obtained:

Long ♀	1,717
Long ♂	1,545
Rudimentary ♀	1,120
Rudimentary ♂	1,179

There is an approach to equality in the last case (1 : .7), and, in consequence, the contrast with the preceding data is striking. For purposes of more detailed comparison the data for the two mass cultures and for the 20 pairs is given:

TABLE I.

BACK-CROSS PAIRS.

No.	Long ♀.	Long ♂.	Rudimentary ♀.	Rudimentary ♂.
7	133	117	94	102
8	59	43	55	47
9	107	114	67	66
10	70	55	46	64
11	62	60	53	36
12	49	36	51	41
13	86	76	31	43
14	135	101	61	104
15	107	102	61	79
16	67	44	60	54
17	116	107	73	70
18	92	80	72	68
19	87	77	84	53
20	16	14	12	6
21	78	101	73	72
22	83	60	44	50
23	88	81	62	91
24	52	49	35	37
25	95	100	56	65
26	135	128	30	31
Total	1,717	1,545	1,120	1,179

Back-cross En Masse.

28	248	235	4	14
29	279	254	3	17
Total.....	527	489	7	31

This same mating was again made both in mass and in pairs. The expectation is equality of long and rudimentary wings. The mass cultures gave:

Long ♀	341
Long ♂	337
Rudimentary ♀	51
Rudimentary ♂	64

When the experiment was made with pairs the following totals were obtained, (with a ratio of 1. : .63):

Long ♀	1,676
Long ♂	1,431
Rudimentary ♀	930
Rudimentary ♂	1,022

For detailed comparison the counts of the two mass cultures and of the 29 pairs taken separately are given in Table II.

Table II.

Back-cross Pairs.

No.	Long ♀.	Long ♂.	Rudimentary ♀.	Rudimentary ♂.
31	161	122	108	154
32	15	7	5	4
33	154	121	27	30
34	82	103	38	66
35	103	81	11	13
36	143	74	12	42
37	86	84	62	49
38	59	79	51	57
39	123	98	67	61
40	164	142	70	85
41	63	59	71	54
42	72	45	47	42
43	56	65	55	55
44	47	51	47	46
45	45	37	34	25
46	93	64	42	47
47	45	50	46	33
48	63	47	44	55
49	59	45	52	65
50	63	57	41	39
Total.....	1,676	1,431	930	1,022

BACK-CROSS EN MASSE.

51	154	135	18	19
52	187	202	33	45
Total...	341	337	51	64

Since rudimentary females are sterile with rudimentary males the stock of rudimentary wings is maintained by breeding rudimentary males to heterozygous, long-winged females.

THE VIABILITY OF THE STRAP-WING RACE.

The peculiarities of structure and inheritance of this mutant will be described in another place. The stock contained "beading" and overlaps in appearance the vestigial wing. The "beaded" flies that appear in F_2 are classified with "normal" or long wing, those with vestigial-like wings are counted in with "strap." Strap bred to wild stock gives sons and daughters with long wings. These bred *en masse* during August gave the following results in eleven cultures. About six to ten F_1 flies were used in each culture in a medium sized bottle, and the food conditions were made as favorable as possible.

	Normal.	Strap.	Ratio.
1.	224	59	1 : 3.8
2.	236	63	1 : 3.7
3.	471 (+39 curved)	28	1 : 17.0
4.	216	45	1 : 4.8
5.	103	11	1 : 9.3
6.	277	24	1 : 11.5
7.	162	14	1 : 11.6
8.	464	23	1 : 20.1
9.	494	82	1 : 6.2
10.	288	44	1 : 6.5
11.	164	34	1 : 4.8
Total......	3,099 (+39)	427	1 : 7.2

The ratios range from 1 : 3.7, to 1 : 20.1. In order to compare these results with matings in pairs, fifteen pairs were mated. After ten days the parents were placed in a new bottle with fresh food and in some cases they were carried to a third bottle. The results are given in the next table (III.).

The results from pairs approximate more nearly to expectation (1 : 3); the totals give a ratio of 1 : 3.57. The individual lots

TABLE III.

F_2 FROM STRAP ♂ × WILD ♀.

No.	Brood I.					Brood II.					Brood III.				
	Normal.		Strap.		Ratio to 1.	Normal.		Strap.		Ratio to 1.	Normal.		Strap.		Ratio to 1.
	♀	♂	♀	♂		♀	♂	♀	♂		♀	♂	♀	♂	
1	97	89	27	17	4.2	218	199	60	47	3.9	—	—	—	—	—
2	112	116	24	20	5.2	148	141	23	26	5.9	48	44	16	14	3.1
3	90	76	13	12	6.6	88	88	8	5	13.5	—	—	—	—	—
4	117	101	37	14	4.3	99	130	38	28	3.5	25	41	10	16	2.4
5	73	63	24	31	2.5	80	88	34	30	2.6	7	3	1	1	—
6	79	90	23	26	3.5	119	100	30	24	4.1	10	14	2	5	—
7	105	86	31	27	3.3	121	115	27	37	3.7	50	59	12	23	3.1
8	94	91	32	22	3.4	120	113	39	36	3.1	24	37	10	6	3.8
9	95	87	19	31	3.6	101	106	29	29	3.6	59	42	17	27	2.3
10	72	60	31	26	2.3	46	59	22	20	2.5	5	—	1	—	—
11	66	63	12	22	3.8	57	36	5	7	7.7	—	—	—	—	—
12	79	68	27	38	2.3	77	77	28	24	3.0	3	3	1	—	—
13	79	65	26	15	3.5	54	62	17	18	3.3	37	29	10	5	4.4
14	85	80	28	26	3.1	78	90	22	21	3.9	32	34	11	7	3.8
15	62	61	14	15	4.1	63	53	21	26	2.5	30	17	7	7	3.4
	1,305	1,196	368	342	3.52	1,479	1,457	403	378	3.75	330	323	98	111	3.12

are worthy of inspection. The ratios run more evenly and range from 1 : 2.3 to 1 : 7.7 with one exceptionally high at 1 : 13.5. The counts from day to day (not recorded here) show that the F_2 strap-winged flies hatch later than the flies with normal wings, so that as the cultures run out, the relative number of strap-wing flies increases, and unless the cultures are exhausted when the count stops there will be a deficiency of strap-wing flies. An attempt was made to run the cultures to a finish, although this cannot always be done. If the cultures dry up, a disproportionately large number of strap-winged flies will be destroyed, and this will account in part for the deficiency in this class. These results are not affected by the age of the parents, as shown by comparison of the ratios in the totals for the second (1 : 3.75) and third (1 : 3.12) broods with that of the first (1 : 3.52).

The above pairs were mated in November and December, 1913. In order to make a more exact comparison, a few mass cultures were made in December from the same stocks. The results are shown in the next table (IV.).

In addition to the preceding data there were other crosses of strap male by wild female that had been made nine months

TABLE IV.

No.	Normal.	Strap.	Ratio.
1	455	67	1 : 6.8
2	365	65	1 : 5.6
3	330	75	1 : 4.4
Total	1,150	207	1 : 5.5

before those recorded above. In these earlier experiments in mass cultures no attempt was made to avoid crowding through use of too many parents, although the bottles were otherwise maintained in good condition. The following F_2 counts were made:

	Long ♀ and ♂.	Strap ♀ and ♂.	Ratio.
1.	739	76	1 : 9.7
2.	411	55	1 : 7.5
3.	247	22	1 : 11.2
4.	410	59	1 : 6.9
5.	464	54	1 : 8.6
6.	266	33	1 : 8.0
7.	490	61	1 : 7.8
8.	342	43	1 : 8.1
9.	137	13	1 : 10.5
10.	493	3	1 : 164.0
11.	245	8	1 : 30.6
12.	197	0	—
13.	818	137	1 : 6.0
14.	614	125	1 : 4.9
Total	5,873	689	1 : 8.53

Whether the two very high ratios under 10 and 12 should be included may be questioned. It is possible though not probable that contamination took place. Excluding these the ratios vary from 1 : 4.9 to 1 : 30.6.

CONCLUSIONS.

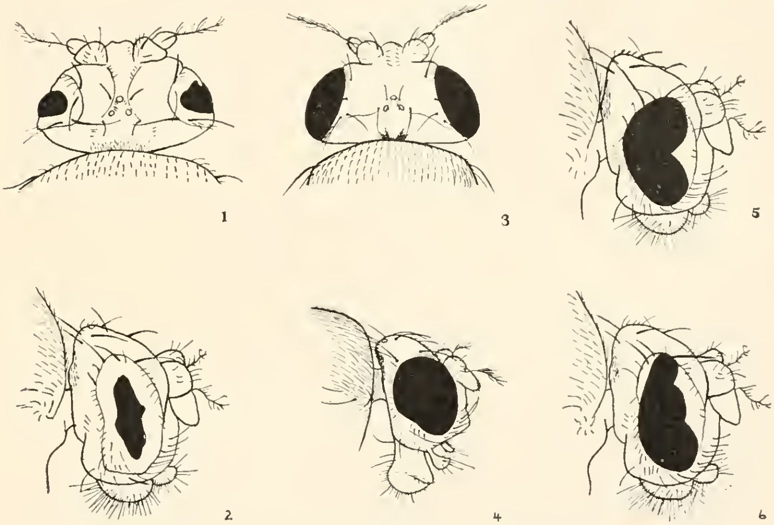
The experiments show that under unfavorable conditions due to crowding the mutant forms, rudimentary wings and strap wings, run behind expectation. That the result is due to crowding is shown when cultures of sister individuals are made *in pairs* with abundant room and food. There is then a closer, and in some cultures a complete agreement with expectation. In experiments in which it is only necessary to show whether a character is recessive or dominant, and whether it is, or is not linked to other characters these deficient ratios, while unfortunate, present no serious difficulties, but in experiments in which it is necessary to determine accurately the linkage ratios the difference becomes serious.

The rudimentary and strap-winged mutants have been used to test the effects of crowding due to mass breeding, because they have been found to show such effects more than any other stocks. In the case of other mutants that give, even in mass cultures, nearly the theoretical values, the method which we now generally employ of breeding by pairs in large culture bottles with plenty of food eliminates almost entirely disturbances due to viability.

A NEW SEX-LINKED CHARACTER IN DROSOPHILA.

SABRA COLBY TICE,

A new sex-linked character (Figs. 1 and 2) recently appeared in *Drosophila ampelophila* in an experiment involving rudimentary and long-winged flies with normally shaped eyes (Figs. 3, 4). The new character "barred" eye appeared in a single male. The ommatidia are reduced in number and are restricted to an area shaped like a vertical bar or band, the edges of which are more or less irregular in shape.



Figs. 1 and 2. Pure barred (male) above and from the side.
Figs. 3 and 4. Normal eye (side view smaller fly).
Figs. 5 and 6. Two heterozygous eyes, both in side view.

Barred Eye by Normal Eye.

When the barred male was bred to normal-eyed females, the F_1 generation showed the barred eye in the females only (Figs. 5 and 6), the males being normal. This is what would be expected if the new character is sex-linked and dominant. The

F_2 generation (Table I.) with class ratios 1 : 1 : 1 : 1 is consistent with this expectation. In the following analyses *Br′* is the factor for the dominant barring and *br′* is its recessive normal allelomorph.

TABLE I.

P_1 Normal ♀, *X br′ — X br′*.
Barred ♂, *X Br′* — ——.

F_1 Females.	F_1 Males.
$\left\{\begin{matrix} X\ br' \\ X\ Br' \end{matrix}\right.$	$\left\{\begin{matrix} X\ br' \\ \text{——} \end{matrix}\right.$
Barred ♀.	Normal ♂.
570	565

Gametes of F_1 barred ♀, *X br′—X Br′*.
Gametes of F_1 normal ♂, *X br′*— ——.

	F_2 Females.		F_2 Males.	
	$\left\{\begin{matrix} X\ br' \\ X\ br' \end{matrix}\right.$	$\left\{\begin{matrix} X\ Br' \\ X\ br' \end{matrix}\right.$	$\left\{\begin{matrix} X\ br' \\ \text{——} \end{matrix}\right.$	$\left\{\begin{matrix} X\ Br' \\ \text{——} \end{matrix}\right.$
No.	Normal ♀.	Barred ♀.	Normal ♂.	Barred ♂.
2	170	136	158	122
3	160	140	142	140
4	155	124	154	119
5	92	105	91	79
6	81	58	87	63
7	112	88	82	83
8	102	86	87	93
9	104	84	92	89
10	87	72	84	90
Total.....	1,063	893	977	878

The barred females of the above F_2 generation were heterozygous in barring, and, when mated to their barred brothers, gave an equal number of barred and normal sons (Table II.).

TABLE II.

Gametes of F_2 barred ♀, *X Br′—X br′*.
Gametes of F_2 barred ♂, *X Br′*— ——.

F_3 Females.	F_3 Males.	
$\left\{\begin{matrix} X\ Br' \\ X\ Br' \end{matrix}\right.$ $\left\{\begin{matrix} X\ br' \\ X\ Br' \end{matrix}\right.$	$\left\{\begin{matrix} X\ Br' \\ \text{——} \end{matrix}\right.$	$\left\{\begin{matrix} X\ br' \\ \text{——} \end{matrix}\right.$
Barred ♀.	Barred ♂.	Normal ♂.
236	106	123

The original barred male was crossed to his daughters (F_1 ♀♀, Table I.) and as expected the results (Table III.) were similar to those of Table II.

TABLE III.

Gametes of F_1 barred ♀, *X br′—X Br′*.
Gametes of P_1 barred ♂, *X Br′*— —— .

Females.		Males.	
X Br′ *X Br′*	*X br′* *X Br′*	*X br′* ——	*X Br′* ——
Barred ♀.		Normal ♂.	Barred ♂.
281		159	140

In the experiments recorded in Tables II. and III. it was noticed that the barring was of two kinds: (1) A very narrow bar (Figs. 1 and 2) which appeared in all barred males, and in about one half of the double class of barred females, and (2) a bar not so narrow which was present in the rest of the females. The loss in the latter case was mainly on the anterior edge and was especially noticeable as a single notch, or two notches, giving the eye a decided heart shape (Figs. 5 and 6). Here also the ommatidia are disturbed from their regular geometrical arrangement, and converge in rows to the notch. The difference in the narrow and broad bars is due to a difference in zygotic composition for, as will be described later, breeding tests have shown that invariably the broader types are heterozygous in barring (*Br′ br′*). When the narrow bar females were bred to

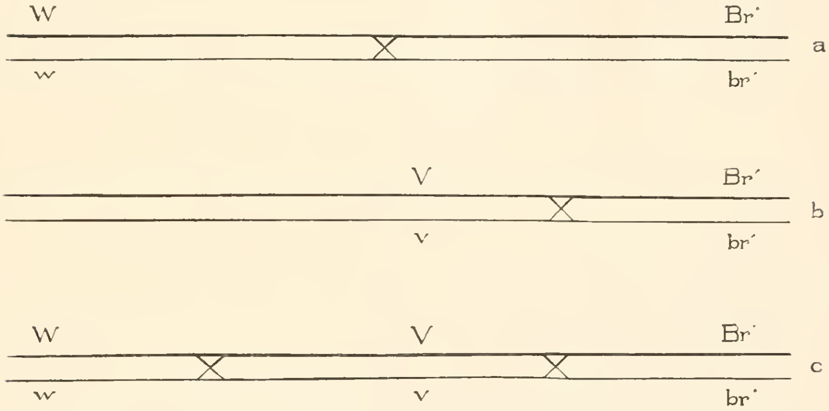


DIAGRAM 1. (*a*) Showing loci of white (*w*) and barred (*br′*) in sex chromosomes of heterozygous female, with the loci of their normal allelomorphs. Crossing over is indicated by the *X* between the lines. (*b*) Ditto for vermilion and barred. (*c*) Ditto for white, vermilion, and barred.

barred males all the offspring were narrow barred. This stock has since bred true.

RED-BARRED EYE BY WHITE NORMAL EYE.

The original barred male was crossed with white-eyed females, with the results given in Table IV. The F_1 ♀ ♀ were red barred, and the F_1 ♂ ♂ were white normal, since white is a sex-linked character. The F_2 generation consisted of four classes, red barred, white barred, red normal, white normal, with an equality of males and females in each. The ratios of the four classes will be discussed later. In the following analyses, *w* is the factor for the recessive white (sex-linked) and *W* is its normal allelomorph (red).

TABLE IV.

P_1 White normal ♀, *X w br′—X w br′*.
Red-barred ♂, *X W Br′*— ——.

F_1 Females.	F_1 Males.
X w br′ *X W Br′*	*X w br′* ——
Red Barred ♀.	White Normal ♂.
483	440

Gametes of F_1 barred ♀ *X w br′—X W Br′—X w Br′—X W br′*.
Gametes of F_1 white ♂—*X w br′*. — ——

	F₂ Females.				F₂ Males.			
	X w br′, *X w br′*,	*X W Br′*, *X w br′*.	*XwBr′*, *Xwbr′*.	*X W br′*, *X w br′*.	*X w br′*, ——	*X W Br′*, ——	*XwBr′*, ——	*X W br′*, ——
No.	White Normal ♀	Red Barred ♀.	White Barred ♀.	Red Normal ♀.	White Normal ♂	Red Barred ♂.	White Barred ♂.	Red Normal ♂.
101	72	84	31	43	86	75	46	71
102	74	58	41	53	65	60	55	52
103	62	81	40	47	57	66	43	52
104	45	35	29	36	21	41	28	30
105	46	58	30	42	35	48	30	46
106	26	44	18	21	24	27	20	29
107	32	44	23	22	25	32	15	19
108	35	43	27	32	31	58	33	45
109	34	41	25	36	31	38	27	39
Total...	426	488	264	332	375	445	297	383

The white heterozygous barred females (F_2 above), bred to their white barred brothers, gave the expected proportions (Table V.).

TABLE V.

Gametes of F_2 white barred ♀, *X w Br′—X w br′*.
Gametes of F_2 white barred ♂, *X w Br′*— ——.

No.	F_3 Females.	F_3 Males.	
	{*X w Br′*, {*X w br′*, {*X w Br′*, {*X w Br′*	{*X w Br′*, { ——	{*X w br′*, { ——
	White Barred ♀ ♀.	White Barred ♂.	White Normal ♂.
a.	198	118	105
b.	224	116	101
Total	422	234	206

By mating together white narrow barred females (homozygous), and white barred males from the above experiment, a white barred stock was obtained which has since bred true.

The original mutant male was mated to his daughters (F_1, Table IV.) and gave the expected proportions (Table VI.).

TABLE VI.

Gametes of F_1 red barred ♀, *X w br′—X W Br′—X W br′—X w Br′*.
Gametes of P_1 red barred ♂, *X W Br′* — ——

Females.	Males.			
Red Barred ♀.	Red Barred ♂.	White Normal ♂.	White Barred ♂.	Red Normal ♂.
240	63	52	50	43

RED BARRED EYE BY VERMILION NORMAL EYE.

The original barred male was mated to vermilion eyed females with the results given in Table VII. As in the two previous cases the barred character was dominant and sex-linked, appearing in the F_1 generation in the females only. The F_1 females, as expected, were red and the males were vermilion, since vermilion also is a sex-linked character. The F_2 generation showed the expected sex-linkage results with four classes: vermilion barred, red normal, vermilion normal, and red barred, with an equality of males and females in each class. The ratios of these four classes will be discussed later.

In the following analyses *v* is the factor for the recessive sex-linked character vermilion and *V* is its normal allelomorph (red).

TABLE VII.

P₁ Vermilion normal ♀, *X v br′—X v br′*.
Red barred ♂, *X V Br′*— ——.

F₁ Females.	F₁ Males.
X v br′	*X v br′*
X V Br′	———
Red Barred ♀.	Vermilion Normal ♂.
367	312

Gametes of F₁ red barred ♀, *X v br′—X V Br′—X v Br′—X V br′*.
Gametes of F₁ vermilion normal ♂, *X v br′*— ——.

	F₂ Females.				F₂ Males.			
	X v br′. *X v br′,*	*X V Br′,* *X v br′.*	*X v Br′,* *X v br′.*	*X V br′,* *X v br′.*	*X v br′,* ——	*X V Br′,* ——	*X v Br′,* ——	*X V br′,* ——
	Vermilion Normal ♀.	Red Barred ♀.	Vermilion Barred ♀.	Red Normal ♀.	Vermilion Normal ♂.	Red Barred ♂.	Vermilion Barred ♂.	Red Normal ♂.
201	315	229	70	95	248	202	80	88
202	284	270	90	102	313	234	82	92
203	250	247	79	103	271	222	68	68
204	93	89	35	24	95	83	34	33
205	99	111	32	38	110	99	45	40
206	72	100	31	31	81	98	20	22
207	108	97	40	57	112	76	43	56
208	141	102	37	46	97	105	36	28
209	125	108	26	28	98	88	26	26
Total...	1,487	1,353	440	524	1,425	1,207	434	453

The heterozygous vermilion barred females (F₂ above) were mated to their vermilion barred brothers and gave the expected ratios (Table VIII.).

TABLE VIII.

Gametes of F₂ vermilion barred ♀, *X v Br′—X v br′*.
Gametes of F₂ vermilion barred ♂, *X v Br′*— ——.

	F₃ Females.	F₃ Males.	
	{*X v Br′,* {*X v Br′,* {*X v Br′.* {*X v br′.*	{*X v Br′,* {———	{*X v br′,* {———
	Vermilion Barred ♀.	Vermilion Barred ♂.	Vermilion Normal ♂.
a.	243	131	124
b.	278	125	169
Total...	521	256	293

By mating vermilion narrow barred females (homozygous) to their vermilion barred brothers, from the above experiment, vermilion barred stock was obtained which has since bred true.

The original male was mated to his daughters (F_1 Table VII.) and gave the expected proportions (Table IX.):

Table IX.

Gametes of F_1 red barred ♀, $X\,v\,br'$—$X\,v\,Br'$—$X\,V\,Br'$—$X\,V\,br'$.
Gametes of P_1 red barred ♂, $X\,V\,Br'$— ——.

Females.	Males.			
Red Barred ♀.	Red Barred ♂.	Vermilion Normal ♂.	Vermilion Barred ♂.	Red Normal ♂.
124	37	48	18	18

The Linkage of the Factor for Barred Eye.

Since the character barred is sex-linked it follows the sex chromosomes. It remains to determine its linkage with other sex-linked factors. Red barred males bred to white normal females gave in the F_2 generation (see Table IV.) the following results:

	White Normal.	Red Barred.	White Barred.	Red Normal.
♀	426	488	264	332
♂	375	445	297	383
Total.....	801	933	561	715

The non-cross over classes are red barred and white normal. The cross over classes are white barred and red normal (Diag. 1*a*). These two categories gives 42.8 per cent. crossing over.

In order to determine the linkage of barred to another sex-linked factor, viz. vermilion; red barred males were bred to vermilion normal females. The F_2 generation (see Table VII.) gave the following results:

	Vermilion Normal.	Red Barred.	Vermilion Barred.	Red Normal.
♀	1,487	1,353	440	524
♂	1,425	1,207	434	453
Total.....	2,912	2,560	874	977

The percentage of crossing over in the above case is 25.3 per cent. Diag. 1*b*.

Earlier experiments (Sturtevant, '13) have shown that there is about 30.7 per cent. of crossing over between white and vermilion. Since there is 25.3 per cent. crossing over between vermilion and barred the factor for barred must lie close to white (approximately 5.4) or else very far from white. That the latter is the case is shown by the large percentage of crossing over between white and barred (42.8 per cent.).

Other work done in this laboratory, largely as yet unpublished, shows that, when distances as long as this between white and barred are involved, the chromosomes often break at two points and re-unite. As a result of this double-crossing over, the non-cross over classes are increased and the cross over classes are diminished (Diag. 1*c*). The amount by which the percentage 42.8 is less than the percentage 30.7 + 25.3 is therefore an indication of the amount of double crossing over that has occurred. Sturtevant ('13) has located certain factors on the X-chromosome. Vermilion (B) is given as 30.7. The experiments in this paper giving 25.3 per cent. crossing over between vermilion and barred enable us to locate the new factor barred approximately at 56 (30.7 + 25.3) (without considering the double crossing-over between white and vermilion or that between vermilion and barred).

DOMINANCE.

In these experiments whenever flies were heterozygous for barring they showed the barring without exception. In this sense the dominance is *constant*, in that a fly which fails to show the barring cannot transmit it. As was stated before the homozygous females have a narrower bar than the heterozygous females. That it is possible to pick out at sight the two different flies is verified by the following experiments.

P_1 Broad Barred ♀ (Heterozygous) by Barred ♂.

F_1	Barred ♀.	Barred ♂.	Normal ♂.
46	80	31	52
47	119	71	72
49	138	80	81
50	101	50	65
51	101	50	56
52	127	75	63
62	94	55	55
65	107	62	48
Total.	867	464	492

P_1 Narrow Barred ♀ (Homozygous) by Barred ♂.

F_1.	Barred ♀.	Barred ♂.
90	65	60
91	43	35
56	86	76
58	101	117
60	118	119
64	90	94
Total.	503	501

P_1 White Broad Barred ♀ (Heterozygous) by White Barred ♂.

F_1.	White Barred ♀.	White Barred ♂.	White Normal ♂.
146	62	30	27
147	86	36	39
151	88	37	44
152	69	31	23
Total.	305	134	133

P_1 White Narrow Barred ♀ (Homozygous) by White Barred ♂.

F_1.	White Barred ♀.	White Barred ♂.
148	81	72
149	83	78
150	45	51
153	105	95
154	63	60
157	38	47
Total.	415	403

P_1 Vermilion Broad Barred ♀ (Heterozygous) by Vermilion Barred ♂.

F_1	Vermilion Barred ♀.	Vermilion Barred ♂.	Vermilion Normal ♂.
249	57	23	29
256	84	39	42
257	67	45	36
258	87	39	37
Total.	295	146	144

P_1 VERMILION NARROW BARRED ♀ (HOMOZYGOUS) BY VERMILION BARRED ♂.

F_1	Vermilion Barred ♀.	Vermilion Barred ♂.
246	67	72
247	30	39
248	52	46
250	42	37
253	43	50
254	48	46
Total	282	290

VIABILITY.

In the foregoing experiment where an equality of barred and normal was expected, it is evident that the mutant character is almost as viable as the normal. For this reason it should prove valuable for further work on linkage. In the experiments in which no linkage is involved, there are 4,671 barred among 9,578 flies. The percentage of barred is 48.7 where 50 is expected. Where the experiments involving linkage are included, the barred flies total 9,767 out of 20,240 and the percentage is 48.2. The relative viability of white and barred appears in those experiments in which both were raised under the same conditions as 45.4 per cent. to 49.9 per cent. The relative viability of vermilion and barred was 51.7 per cent. to 46.8 per cent.

CONCLUSION.

Barred eye—a new sex-linked character—appeared in *Drosophila* in a single red-eyed male fly and its dominance is constant over the normal eye. The broad bar of the heterozygous females distinguishes them from the homozygous ones with a narrow bar. The viability of the stock makes it valuable for linkage experiments.

By taking the percentage of cross overs as an indication of distance on the chromosome, we can place the factor for barred approximately at 56.

ZOÖLOGICAL LABORATORY COLUMBIA UNIVERSITY,
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ANOTHER CASE OF MULTIPLE ALLELOMORPHS IN DROSOPHILA.

T. H. MORGAN.

It has been shown[1] that the factor for white eye color and the factor for eosin eye color are allelomorphs. Each arose as a mutation, but unlike other original and independent mutations they do not give, when crossed, the wild type (red eye) by recombination. Each factor has the same normal allelomorph in the wild fly, and each factor gives the same linkage ratio as the other one with any sex-linked factor. The factor for eosin, the factor for white and their normal allelomorph form a system of triple allelomorphs. Only two of these factors can exist at any one time in the same female, and, since they are sex-linked, only one at a time in a male. In consequence, a red-eyed female may be heterozygous for white or for eosin, but never for white and for eosin.

Another similar case has also appeared. In certain crosses, involving yellow body color, a yellowish male appeared that was characterized by a light spot on the upper surface of the posterior end of the abdomen (Figs. 3 and 4). When the yellow stock was examined another male of the same kind was found there. It is evident that in the first case the new type must have come from the original yellow stock. The mutation probably occurred in the stock one or more generations before it was discovered.

It was natural to assume that such a peculiar spot could be transferred to gray or to black flies. The original male and later many of his offspring that bore the same character were mated to gray and to black flies. The first generation was always normal gray. In the second generation the yellows and the yellow blacks ("browns") were spotted, but never any of the gray or the black flies. Thinking that the spotting was closely linked to the yellow factor and might cross over if enough flies were bred I continued the experiment for four or five months.

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[1] Morgan and Bridges, *Journ. Exp. Zool.*, XV., 1913.

While no record of the number of flies that were examined was kept, I think more than 30,000 gray and black flies must have been looked over. In no case was the spot present.

The alternatives then are absolute (or possibly nearly absolute) linkage, or a case of multiple allelomorphism. In practice these alternatives would give the same numerical results. The theoretical differences between the idea of absolute linkage and multiple allelomorphism will be discussed later.

When a spot male was mated to wild females, all the offspring were normal gray without spot. When the F_1 offspring were inbred the following classes were produced.

	Gray ♀.	Gray ♂	Spot ♂.
a.	285	132	94
b.	305	127	113
Total...........	590	259	207

There is one class of females and two classes of males. This is the expectation if spot is due to a sex-linked factor. It is the same kind of result that obtains when a yellow male is crossed to a wild female. When an F_1 female from the last experiment was back crossed to a spot male the following results were obtained:

	Gray ♀.	Spot ♀.	Gray ♂.	Spot ♂.
a.	100	88	91	84
b.	25	16	15	20
c.	86	87	70	48
Total.....	211	191	176	152

Equality in all four classes is expected and this is practically realized. The spot type seems somewhat less viable than the wild type. The male counts run behind the female, which is a usual result. Here the spot also occurs in the female (Figs. 7 and 8).

A spot female was mated to a yellow male. The 63 daughters were yellow without spot which shows that spot is completely recessive to yellow. The 58 sons were spot, receiving their sex chromosome from the mother. When a pair of these F_1 flies were bred, the following F_2 classes were produced.

Yellow ♀.	Spot ♀.	Yellow ♂.	Spot ♂.
44	45	41	45

This is the expected result for this combination.

The body-color black belongs to our second group of linked factors. A spot female was mated to a black male. The daughters were gray without trace of spot, the sons were spot. Female producing sperms bring in the normal allelomorph for spot (Y), which, with the normal allelomorph for black (B), gives gray. The sons are spot because the male receives his sex chromosome from his mother. The formulas for the F_1 and F_2 generations are the following:

Spot ♀, $y_sB — y_sB$.
Black ♂, Yb —— b.

F_1 $\begin{cases} \text{Gray ♀}, & y_sB\ Yb. \\ \text{Spot ♂}, & y_sB — b. \end{cases}$

$y_sB — Yb — y_s b — YB$, Eggs of $\}$ F_1.
$y_sB — y_sb$ —— B —— b, Sperm of $\}$ F_1.

y_sBy_sB,	spot.	$y_sB — B$,	spot.
Yby_sB,	gray.	$Yb — B$,	gray.
y_sby_sB,	spot.	$y_sb — B$,	spot.
YBy_sB,	gray.	$YB — B$,	gray.
y_sBy_sb,	spot.	$y_sB — b$,	spot.
Yby_sB,	black.	$Yb — b$,	black.
y_sby_sb,	spot black.	$y_sb — b$,	spot black.
YBy_sb,	gray.	$YB — b$,	gray.

The F_2 expectation is 3 gray, 3 spot, 1 black, 1 spot black, and is the same for the male and the female classes which are counted together in the following F_2 results for this cross.

F_2 Females.		F_2 Males.	
Gray.	Spot.	Black.	Spot Black.
133	90	47	39

The double recessive is spot black, which corresponds in color to yellow black or "brown" of earlier papers. The light spot shows here even more strikingly, because of contrast, than in the simple "spot" itself.

Pure stock of the double recessive spot black being on hand, a male was crossed to a yellow female. The F_1 were yellow flies, 65 males and 20 females without spot, showing again the dominance of yellow to spot in the "compound" y-y_s. These F_1 flies inbred gave:

Yellow ♀.	Yellow Black ♀.	Yellow ♂.	Yellow Black ♂.	Spot Black ♂.	Spot ♂.
126	37	33	26	11	26

A male of the spot black stock was also bred to a black female. The offspring were black males and females, which inbred in four lots gave the following results:

	Black ♀.	Black ♂.	Spot Black ♂.
1.	313	165	109
2.	291	140	75
3.	396	211	85
4.	494	255	144
5.	363	178	115
Totals.	1,857	949	528

In these mass cultures, although an attempt was made to have the food conditions favorable, yet, owing to crowding, the spot black males ran far behind the black males.

There appeared in the above F_2 a yellow black fly with no trace of spot. Since in some cultures (but not here) the spot may be faint and difficult to detect with certainty, it seemed possible that the fly was such a *somatic* variant. He was tested by breeding to a black female. The offspring were black (very few in number). These inbred gave:

	Black ♀.	Black ♂.	Yellow Black ♂.
1	55	19	32
2	316	172	151
3	59	42	29
Total.	430	233	212

Had the male in question been only a somatic condition of yellow (suppressed spot) we should have expected some spot males in the F_2 generation. Since none at all appeared there can be no doubt that the grandparent was in reality a *yellow* black individual both somatically and gametically. The most probable explanation is that a mutation backwards from spot to yellow took place (with no change in the black). Another interpretation is that contamination had occurred. This seems highly improbable since at the time there was no stock of yellow black in the laboratory, and only a few experiments under way in which such individuals were being produced. Nevertheless, so long as there were no other characters than these in the experiment by means of which the offspring could be further identified, I think that one cannot be too careful in interpreting such rare occurrences as mutation backwards. A third possibility is that a cross over occurred, and if so, spot is closely linked to yellow, and crossed over only in this one case out of the many thousands of cases.

In order to see whether the spot would show in the female of the "compound" yellow-spot, when the compound was also black, a spot black female was bred to a yellow black male. All the daughters were yellow black without trace of spot. The sons were spot black. These mated together gave the following F_2 generation.

	Yellow Black ♀.	Spot Black ♀.	Yellow Black ♂.	Spot Black ♂.
1.	15	29	18	25
2.	45	47	42	25
3.	60	62	61	51
Totals....	120	138	121	101

OTHER CROSSES WITH SPOT.

We have another stock of black-colored flies called ebony which belongs to our third group of factors. When spot female was crossed to ebony male the daughters were gray and the sons were spot. These inbred gave in F_2 the following results.

Gray.	Spot.	Ebony.	Spot Ebony.
91	102	29	24
106	73	45	28

The analysis is the same as in the case of spot by black. The double recessive is spot ebony which has a brownish color differing in tint from the brown of spot black.

There is still another black-colored mutant called sable. It is sex-linked, and belongs, therefore, in our first (or sex-linked) group of factors. When a spot female was bred to sable male the daughters were gray and the sons spot. Inbred these gave the following results.

Gray ♀.	Spot ♀.	Spot ♂.	Sable ♂.	Gray ♂.	Spot Sable ♂.
35	30	17	20	24	8

The analysis is the same as when the cross is made with yellow, except that gray is formed by recombination here. The linkage of spot and sable modifies the ratios somewhat.

In the cross with ebony and in that with sable there appeared in the F_1 generation a few exceptional cases that are due to non-disjunction. The actual results are

	Spot ♀.	Gray ♀.	Spot ♂.	Gray ♂
F_1	0	112	104	3
	0	66	52	4

	Spot ♀.	Gray ♀.	Spot ♂.	Sable ♂.
F_1	2	62	64	2

These cases are like those described by Bridges and call for no further comment here. They are explicable on the assumption that the two sex chromosomes of certain spot females occasionally stick together, and either pass out of the egg or else both remain in it. In the first work done more than a year ago with spot, some unexplained results were obtained but not then interpreted. It now appears that in part at least they may have been due to non-disjunction.

The Origin of Multiple Allelomorphs.

It is noteworthy that spot appeared in yellow stock. It may seem that there is some causal connection here since spot is a mutation in the same locus as yellow. Likewise eosin appeared in white stock, and is another case of multiple allelomorphism. Plausible as is the assumption it can not be proven, but the following considerations are not without interest. If the mutation appeared first in the yellow stock, in a chromosome that went into a male, the spot would be apparent on examination. If the chromosome went into a female the mutation would not appear for another generation and then in a male. If on the other hand the mutation had appeared in a chromosome in the gray stock it might appear in the next generation if it went into a male, just as yellow itself might have appeared in the same way. If the chromosome in question had passed into a female no evidence of the mutation would be seen, but it would appear in the second generation males. The two cases are alike. Since, however, the mutation has never appeared in the gray stock, although a great many more gray flies have been seen than yellow flies, it might be argued that a mutation in a locus is more likely to be followed by another in the same locus than if no mutation had taken place there. But mutations are such rare phenomena that this argument does not carry much weight, and judgment must be suspended until further mutations have shown themselves.

OTHER CHARACTERISTICS OF SPOT.

In general we are apt to seize upon the most evident characteristic of a mutation, give it a name, and neglect to mention, or overlook, other effects that may be associated with it. In the case of the white eye mutation for instance the eye attracts our attention, yet if red-eyed and white-eyed flies be put into alcohol the yellow, especially the yellow bands, become markedly different in the two flies. The white-eyed flies show much whiter bands. Evidently the mutation has affected other pigments than those contained in the eye.

In another mutation called club wing (as yet undescribed) the wing pads of many flies fail to expand, although others do expand even in pure stock. It was later found that a small bristle on each side of the thorax is absent in the club stock irrespective of whether the wing pads expand or not. This microscopic change is a constant feature of the mutation and a better character for that reason than the more obvious although inconstant one of the club-shaped wing.

Likewise in "spot." In the black spot fly there is a distinct light dot on the post thorax, and often a light band down the middle of the thorax (Fig. 4a). These were called dot and dash, but it was later noticed by Bridges that the dot and dash are present in flies only for a short time and disappear as the flies get older. All attempts to get stocks with permanent dot and dash failed, because, as we now think, these are only juvenile characters. The dot is sometimes seen in young spot flies, but is weak and soon disappears. Dot and dash are, therefore, due to the same mutation that gave the spot, but are ephemeral and are especially seen in the double recessive condition—black spot. The different degrees of viability shown by the different mutations may also be looked upon as effects associated with the mutation—effects of the highest importance in determining the chances of survival of the mutants, but of small value as indicators of the mutation itself.

Linkage of Spot and White.

If, as the evidence shows, spot is an allelomorph of yellow, and both have a common normal allelomorph, it was not considered worth while to carry out any extensive experiments in linkage, since the data for yellow would apply to spot. Incidentally, however, a few experiments were made with white-eyed flies, and may be given here.

A few yellow white males were bred to spot red-eyed females. The F_1 flies were yellow red females and spot red males. These inbred gave:

	Yellow Red ♀.	Spot Red ♀.	Yel. White ♂.	Spot Red ♂.	Yel. Red ♂.	Spot White ♂.
(1)	63	19	40	31	0	1
(2)	94	21	47	60	1	0

The linkage of spot and white shown by these figures is 1.11. That already determined for yellow white is 1.12. The coincidence is greater than might be expected with small numbers for loci so near together.

In another experiment a spot black, red-eyed male was mated to a yellow white-eyed female. The daughters were yellow red, the sons yellow white flies. These inbred gave:

Females.

Yellow Red.	Yellow Black Red.	Yellow White.	Yellow Black White.
45	11	41	9

Males.

Spot Red.	Spot-black Red.	Yellow White.	Yellow-black White.
39	13	33	12

The analysis follows:

Yellow white ♀, $ywBywB$.
Spot black ♂, y_sWbb.

$$\left.\begin{array}{l} ywB\text{— }ywB,\ \text{Eggs of} \\ y_sWb\text{— —}b,\ \text{Sperm of}\end{array}\right\}P_1.$$

F_1 ♀, $ywBy_sWb$.
F_1 ♂, ywB—b,
Eggs yWb—y_sWB—yWB—ywB—y_sWb—y_swb—ywb—y_swB,
Sperm ywB—ywb— —B— —b,

F_2 Females.		F_2 Males.	
$yWbywB$,	yellow red.	yWb —B,	yellow red.
y_sWBywB,	yellow red.	y_sWB—B,	spot red.
$yWBywB$,	yellow red.	yWB —B,	yellow red.
$ywBywB$,	yellow white.	ywB —B,	yellow white.
y_sWbywB,	yellow red.	y_sWb —B,	spot red.
y_swbywB,	yellow white.	y_swb —B,	spot white.
$ywbywB$,	yellow white.	ywb —B,	yellow white.
y_swBywB,	yellow white.	y_swB —B,	spot white.
$yWbywb$,	yellow black red.	yWb —b,	yellow black red.
y_sWBywb,	yellow red.	y_sWB—b,	spot red.
$yWBywb$,	yellow red.	yWB —b,	yellow red.
$ywBywb$,	yellow white.	ywB —b,	yellow white.
y_sWbywb,	yellow black red.	y_sWb —b,	spot black red.
y_swbywb,	yellow black white.	y_swb —b,	spot black white.
$ywbywb$,	yellow black white.	ywb —b,	yellow black white.
y_swBywb,	yellow white.	y_swB —b,	spot white.

As shown by the preceding analysis, the expectation, without considering the strong linkage between spot and white, is as follows:

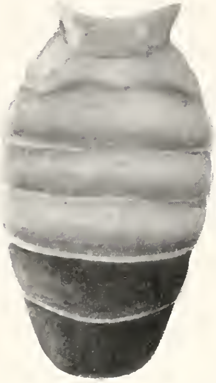
Females.	
Yellow red	6
Yellow white	6
Yellow-black red	2
Yellow-black white	2

Males.	
Yellow red	3
Yellow white	3
Yellow-black red	1
Yellow-black white	1
Spot red	3
Spot white	3
Spot-black red	1
Spot-black white	1

The four classes of females were realized in the results, but only four of the possible eight classes of males appeared. These four are the non-cross-over classes between spot and white; those absent are the crossovers. Considering the small numbers involved, the absence of these four classes of males is a probable event.

EXPLANATION OF PLATE I.

FIG. 1. Normal abdomen of male, dorsal view.
FIG. 2. Ditto, side view.
FIG. 3. Spot abdomen of male, dorsal view.
FIG. 4. Ditto, side view.
FIG. 4*a*. Thorax (dorsal view) of young spot black showing dot and dash.



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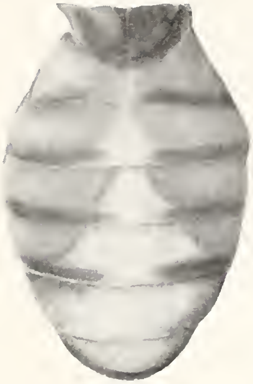

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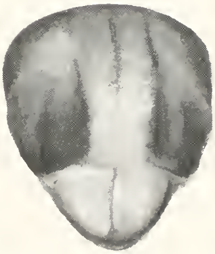

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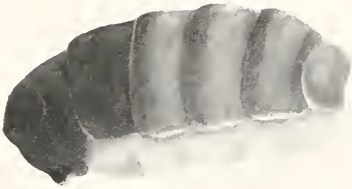

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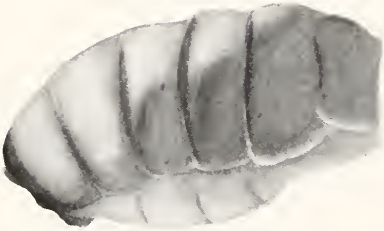

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MORGAN.

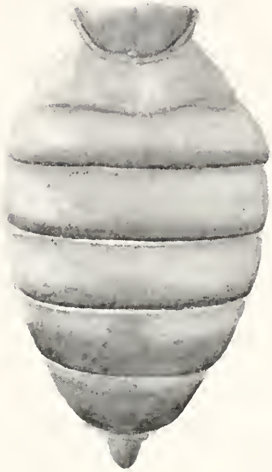
EXPLANATION OF PLATE II.

FIG. 5. Normal abdomen of female, dorsal view.

FIG. 6. Ditto, side view.

FIG. 7. Spot abdomen, dorsal view.

FIG. 8, Ditto, side view.



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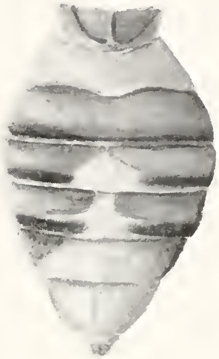

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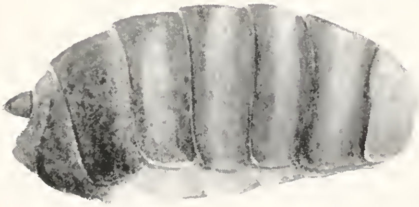

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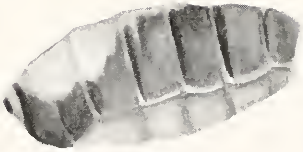

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MORGAN.

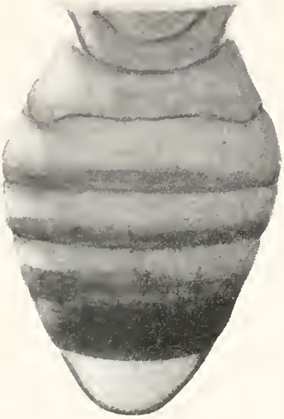
EXPLANATION OF PLATE III.

FIG. 9. Spot abdomen of male showing slight development of spot, dorsal view.

FIG. 10. Ditto, side view.

FIG. 11. Ditto, female, dorsal view.

FIG. 12. Ditto, side view.



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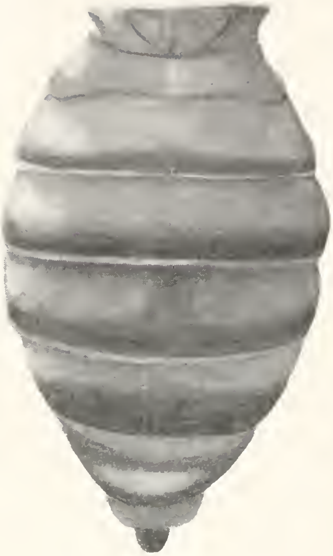

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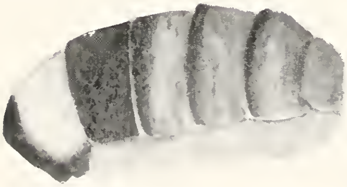

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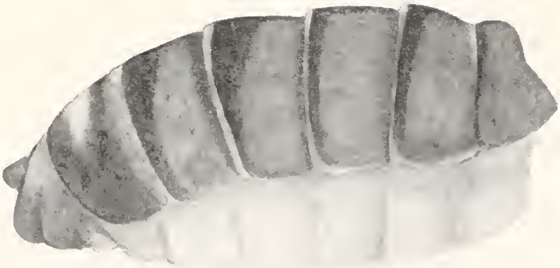

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Vol. XXVI. *May, 1914.* *No. 5*

BIOLOGICAL BULLETIN

AN EXPERIMENTAL STUDY OF CONCRESCENCE IN THE EMBRYO OF CRYPTOBRANCHUS ALLEGHENIENSIS.[1]

BERTRAM G. SMITH.

I. Introduction.

The concrescence theory is an attempt to establish a universal law for the formation of the embryo. It is also an attempt to apply to conditions found in the vertebrates, a law having a wide range of validity in the invertebrates.

In its widest sense, concrescence may be defined as the building-up of the body of the embryo by the union along the median line, of parts that are previously laid down as separate bilateral foundations. The classical case is that of the leech (Whitman, '78), in which the entire body, excepting the head, is formed by concrescence.

In the vertebrates the formation of the nerve tube by the apposition of the neural folds, and the formation of the gastral mesoderm in two more or less distinct halves which later unite across the median line, come within the category of concrescence in this wide sense of the term. But the problem has usually been restricted to certain aspects of development intimately related to gastrulation. Thus, in the teleost the convergence[2] of the germ ring until its materials coalesce to form the posterior part of the embryo, has been regarded as a process of concrescence. A very similar process occurs, though less conspicuously, in the amphibian embryo.

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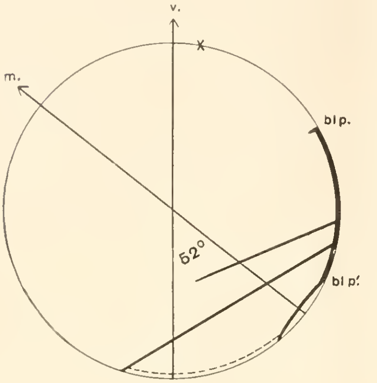
[1] An abstract of this paper was read before the American Society of Zoologists at the meeting in Philadelphia, December 29, 1913.

[2] Since among authors there is no uniformity in the use of the words *convergence, confluence* and *apposition* in their relation to concrescence, I have felt free to use each term in the sense that seems to me the most appropriate.

There remains a more problematical phase of concrescence which has been studied in various vertebrates, and which I have recently investigated in the amphibian embryo. The question involved is this: during the process of overgrowth of the yolk by the dorsal lip of the blastopore, is material dorsal to the blastopore carried from both sides toward the median line? In other words, is there a confluence of material in the median line of that part of the embryo that is formed by overgrowth



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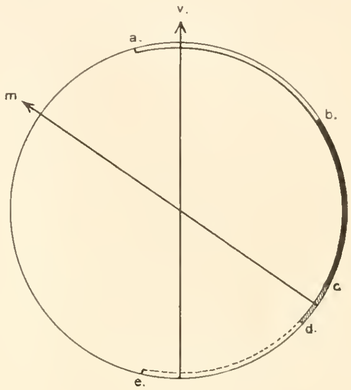

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FIG. 1. Diagram showing in lateral view successive positions of the blastopore in the gastrula of *Cryptobranchus allegheniensis*. *blp.* and *blp'.* respectively indicate early and late positions of the dorsal lip of the blastopore; *m* is the morphological axis of the egg, and *v* the vertical axis determined by gravity. The cross indicates the position of the anterior end of the neural groove.

FIG. 2. Diagram showing in lateral view the position of the embryonic body, and illustrating some features of embryo-formation, in the egg of *Cryptobranchus allegheniensis*. *a* to *b* (72°), the portion of the embryo formed in situ; *b* to *c* (60°), the portion of the embryo formed by overgrowth of the dorsal lip of the blastopore; *c* to *d* (about 16°), portion formed by convergence of the lateral and ventral lips of the blastopore; *d* to *e* (60°), distance travelled by the ventral lip of the blastopore. Other lettering as in the preceding figure.

and in-turning of the dorsal lip of the blastopore? The problem as concerns the egg of *Cryptobranchus* may be more clearly pointed out with the aid of the accompanying figures (Figs. 1 and 2). In Fig. 1 the transverse lines indicate successive positions of the blastopore, viewed from the lateral aspect. In Fig. 2 the relative amounts of the embryo formed in different ways are represented: about 72° of the anterior end of the embryo is formed in situ, so far as overgrowth and convergence are concerned; about 16° of the posterior end of the embryo is formed by the convergence of material lying in the region of the lips of the blastopore; the remaining middle portion of the embryo, about 60° in extent, is formed in connection with overgrowth by the dorsal lip of the blastopore, with the possibility of confluence. It is the possibility of confluence in this region that we desire to investigate; also some details concerning convergence and the movements of the neural folds.

The concept of concrescence, especially in the earlier stages of its history, has often been assumed to involve the idea of a certain amount of preformation in the parts that are brought together; they must be differentiated as the anlage of definite organs. In the present paper I shall neglect entirely this phase of the problem; the term concrescence is here employed without any implications as to the amount of differentiation in the regions concerned.

II. Methods and Technique.

Nearly all the previous experimental work on the problem of concrescence has been done by mechanical methods: an injury of some sort has been inflicted on the living egg and the result in later stages noted. The usual mode of procedure has been to prick the egg with a fine needle, making a slight puncture sufficient only to establish a landmark by means of which the movement of materials may be followed.

The present study is an attempt to apply the method of vital staining to the solution of this problem. The advantage of this method is that extensive markings may be made without in the least interfering with the normal course of development. From Goodale ('11) was obtained the idea of using Nile blue sulphate for this purpose. On account of marked differences in the structure of the gelatinous envelopes of the eggs of the two species, the method of applying the stain to the egg of *Cryptobranchus* necessarily differs from that employed by Goodale for the egg of *Spelerpes*.

Nile blue sulphate in aqueous solution of the proper strength produces on the egg or embryo of *Cryptobranchus* a very distinct spot which will not wash out, and which is not toxic enough to interfere with the normal development. The large size of the egg (from 6 to 7 mm. in diameter), the ease with which it may be removed from its gelatinous envelope, and the entire absence of pigment make it a peculiarly favorable object for staining experiments. From the behavior of the stain in experiments it appears that it does not spread to any perceptible extent by diffusion, and that stained areas are carried from one position to another only by an actual movement of material. A very faint trail may sometimes be left behind a moving spot, but this appears to be due to secondary staining by the vitelline membrane. The stain enables us to determine the direction and amount of the movement of cells, and to distinguish an actual transference of cellular material from a wave movement or undulation.

My results with *Cryptobranchus* do not harmonize with Goodale's ('11) statement that in *Spelerpes* the yolk granules only are stained. In the early cleavage stages of *Cryptobranchus* the micromeres stain distinctly and keep the stain, while the more heavily yolk-laden parts of the egg stain with difficulty and the stain more readily washes out. In later cleavage stages the micromeres stain more intensely than in the early stages, and the neural folds take the stain with even greater intensity. The inference would seem to be that the cytoplasm stains more readily than the yolk.

The method used in applying the stain to the egg of *Cryptobranchus* is as follows: The egg is removed from its gelatinous envelope and placed in water in a Syracuse watch glass. A small drop of strong aqueous solution of Nile blue sulphate is applied to its surface with a fine pipette. After an interval of about half a minute the excess of stain is washed away and sucked up with a clean pipette. On account of the large size of the egg, magnification is not necessary. The under surface of the egg may be viewed by means of a mirror placed under the watch glass; the mirror is far enough away from the egg so that the entire image may be observed at a single view. A lateral view of the egg may be obtained by holding a piece of mirror in a vertical position in the watch glass beside the egg. For purposes of observation it is possible to turn the egg with a camel's hair brush, and keep it in any desired position on a bed of cotton; but on account of the extreme delicacy of the egg, mechanical manipulation is preferably to be avoided. The drawings used in illustrating this paper were made with the aid of a mirror. A camera lucida was not employed, for the reason that its use requires too much handling of the egg in order to get it in the right position for drawing. Free-hand sketches were made as accurately as possible, and it is believed that they give as faithful pictures as would be the case were they made with a camera.

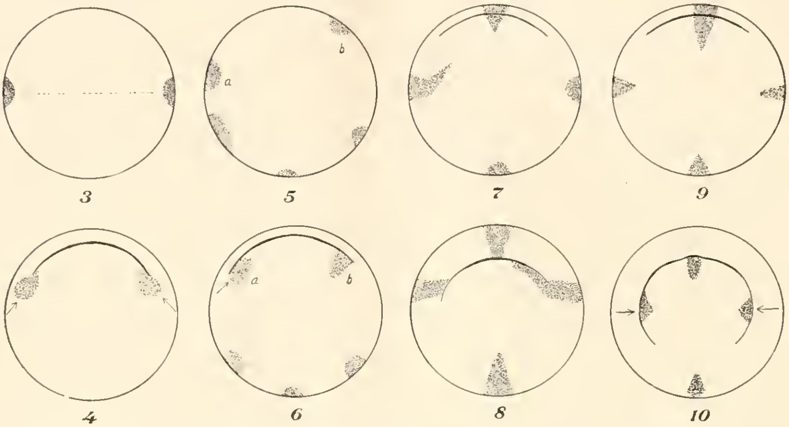
III. Experiments and Observations.

A. Convergence.—In my previous work on the development of *Cryptobranchus* (Smith, '12), observational studies alone gave evidence that a band of cells occupying an equatorial position in the late blastula moves, during gastrulation, toward the vegetal pole and converges on the site of the closing blastopore to form the posterior end of the embryo. By means of staining experiments Goodale ('11) has demonstrated a similar process in the eggs of *Spelerpes* and *Amblystoma.* The following experiments enable us to determine more precisely the nature of this movement and the distribution of the cells involved.

In connection with another series of experiments some eggs were stained in the early first cleavage stage by placing a mark in the equatorial region opposite each end of the cleavage furrow. Figs. 3 and 4 represent one of these eggs in which the first cleavage furrow extended precisely at right angles to the median plane of the future gastrula. In Fig. 4, representing the lower hemisphere of the early gastrula, the two spots have been carried ventrally and posteriorly to a position at the ends of the crescentic blastopore, which they tend to enter from below (*i. e.*, from the posterior side). In comparing these and similar figures, it must be remembered that, since the egg is a spherical object and the drawing a projection on a plane surface, the distance through which a mark travels is in certain situations much greater than appears in the drawings.

Fig. 5 represents the lower hemisphere of an egg stained in the equatorial region of the late blastula. Fig. 6, drawn two days later, shows the spots on the posterior side of the egg appearing entirely below the dorsal lip of the blastopore. They have also moved toward the median line.

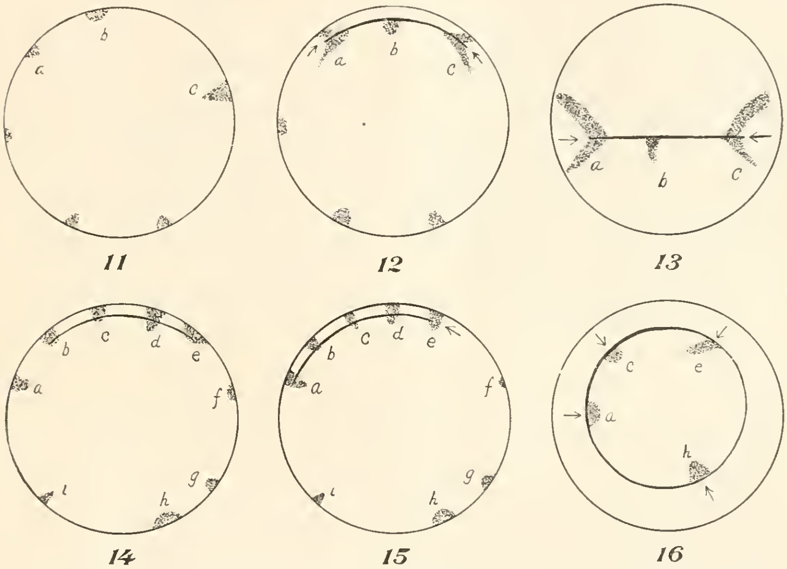
Fig. 7 represents the lower hemisphere of an early gastrula stained in the equatorial region in such a way that one of the spots extends in a meridional direction across the dorsal lip of the blastopore. The results two days later (Fig. 8) demonstrate overgrowth and convergence.



FIGS. 3 TO 10. Each vertical pair of figures represents the history of an individual egg of *Cryptobranchus alleghenicnsis*. The dotted areas indicate spots produced with Nile blue sulphate. Fig. 3, lower hemisphere of an egg in the first cleavage stage, sketched immediately after marking in the equatorial region opposite the ends of the cleavage furrow. The dotted line indicates the position of the first cleavage furrow which is confined to the upper hemisphere. Fig. 4, lower hemisphere of the same egg sketched a week later in the early gastrula stage. Fig. 5, lower hemisphere of an egg in a late blastula stage sketched immediately after marking in the equatorial region. Fig. 6, lower hemisphere of the same egg, sketched in the early gastrula stage four days later. Fig. 7, lower hemisphere of an egg in the early gastrula stage, sketched immediately after marking in the equatorial region. Fig. 8, lower hemisphere of the same egg sketched two days later. Fig. 9, lower hemisphere of an egg in the early gastrula stage, sketched immediately after marking in the equatorial region. Fig. 10, lower hemisphere of the same egg sketched three days later.

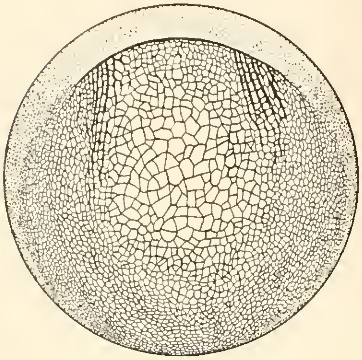
Fig. 9 represents an egg treated in approximately the same manner as the one shown in Fig. 7. This egg sketched three days later demonstrates the in-turning of a large area of the former dorsal lip of the blastopore, and shows more clearly than the preceding the phenomena of convergence. In addition it shows that convergence involves, not merely the lips of the blastopore itself, but a broad zone of cells occupying the lateral margin of the yolk plug.

Fig. 11 represents the lower hemisphere of an egg stained in the equatorial region of the late blastula. The stained areas are elongated in a meridional direction. Figs. 12 and 13 show the condition two days later. At the level of the early blastopore there is a pronounced shifting of material from each side toward the median line.



Figs. 11 to 16. Each horizontal row of figures represents the history of an individual egg of *Cryptobranchus allegheniensis*. The dotted areas indicate spots produced with Nile blue sulphate. Fig. 11, lower hemisphere of an egg in a late blastula stage, sketched immediately after marking in the equatorial region. Fig. 12, lower hemisphere of the same egg sketched in the early gastrula stage, two days later. Fig. 13, same as the preceding, posterior view. Fig. 14, lower hemisphere of an egg in an early gastrula stage, sketched immediately after marking in the equatorial region. Fig. 15, lower hemisphere of the same egg, sketched a day later. Fig. 16, lower hemisphere of the same egg sketched two days later than the preceding.

Fig. 14 represents the lower hemisphere of an egg stained in the equatorial region of the early gastrula; some of the spots extend in a meridional direction across the blastopore. Fig. 16, sketched three days later, shows marked overgrowth and in-turning on all sides of the yolk plug and particularly on the dorsal side. In addition this figure shows that convergence involves a zone of cells occupying the ventral as well as the lateral margin of the yolk plug. This figure in connection with Fig. 10 shows that the movement of the ventral margin occurs considerably later than the movement of the lateral margin—a result correlated with the delayed development of the ventral lip of the blastopore.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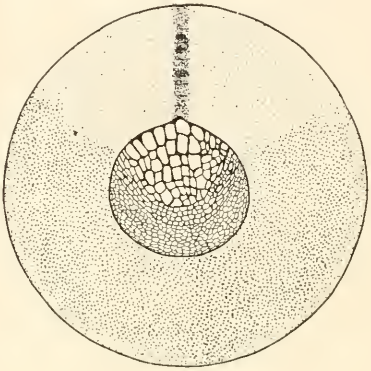
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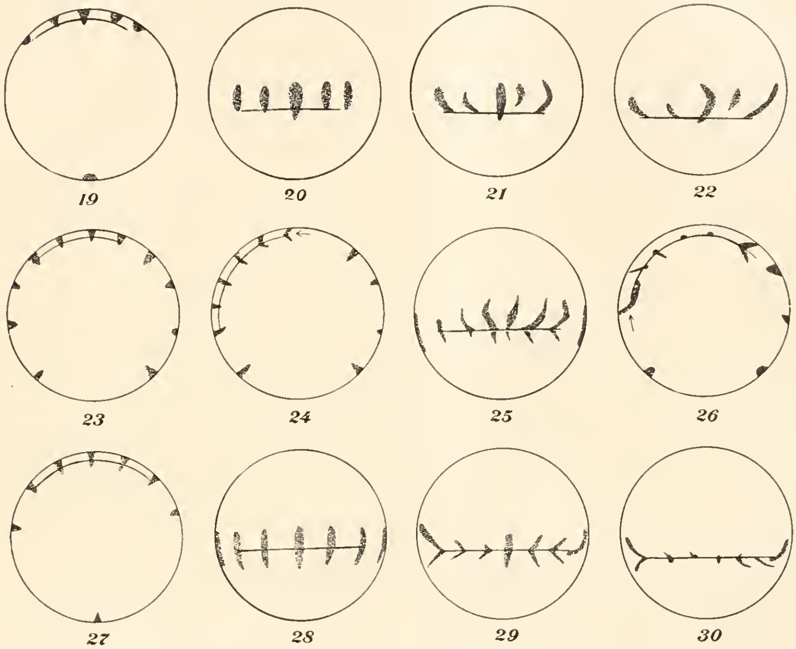
Fig. 17. Lower hemisphere of an early gastrula of *Cryptobranchus alleghenienis*, showing cell outlines. × 7.

Fig. 18. Posterior view of an embryo of *Cryptobranchus alleghenienis* in the neural groove stage, showing cell outlines in the yolk plug. × 7.

Figs. 17 and 18 show that the cells occupying the lateral and ventral margins of the yolk plug, which figure so conspicuously in these movements, are readily distinguishable from the larger yolk cells on the one hand, and the smaller cells of the lips of the blastopore on the other; these marginal cells are arranged in the form of a crescent. We have shown that the marginal cells are the descendents of cells that formed a band in the equatorial region of the late blastula, and it may be here stated that these equatorial cells were of a transitional character, intermediate in size between micromeres and macromeres.

B. Confluence.—The experimental study of the movement of material in the region of the dorsal lip of the blastopore aims to determine (*a*) whether movements other than overgrowth and in-turning occur in the dorsal lip of the blastopore, and (*b*) if such movements occur, can they be explained as a mechanical necessity in the process of invagination and epiboly? My earlier observations (Smith, '12) gave no clue to the occurrence of confluence in this region, so that I approached the experimental



FIGS. 19 TO 30. Each horizontal row of figures represents the history of an individual egg of *Cryptobranchus allegheniensis.* The dotted areas indicate spots produced with Nile blue sulphate. Fig. 19, lower hemisphere of an early gastrula sketched immediately after marking in the equatorial region, especially above the blastopore. Fig. 20, the same, posterior view. Fig. 21, a posterior view of the same egg, sketched a day later. Fig. 22, posterior view of the same egg sketched a day later than the preceding. Fig. 23, lower hemisphere of an early gastrula sketched immediately after marking in the equatorial region. Fig. 24, a similar view of the same egg sketched a day later. Fig. 25, the same as the preceding, posterior view. Fig. 26, the lower hemisphere of the same egg sketched a day later than the preceding. Fig. 27, lower hemisphere of an early gastrula sketched immediately after marking in the equatorial region. Fig. 28, the same, posterior view. Fig. 29, posterior view of the same egg, sketched a day later than the preceding. Fig. 30, posterior view of the same egg, sketched a day later than the preceding.

study of the subject in a skeptical frame of mind that made the results the more impressive, since they were entirely unexpected.

Figs. 19 and 20 represent an egg stained in the equatorial region of the early gastrula. Fig. 21, sketched a day later, shows unmistakably that, during the process of overgrowth, material lying immediately dorsal to the blastopore is being carried from both sides toward the median line—a phenomenon which I have called "confluence." Fig. 22, sketched two days later than the preceding, shows the same process carried further, with the complication that the upper ends of the most laterally situated marks are being carried downward and even slightly outward through being involved in the process of convergence. The outward movement is more readily comprehensible if we compare the homologous regions in the embryos of an elasmobranch and a teleost.

Figs. 23 to 26 represent the history of another egg stained in the same manner as the preceding. The latest stage figured shows incidentally that overgrowth is accompanied by extensive in-turning of the dorsal lip of the blastopore. The results as regards confluence are even more striking; but the figures give some evidence of a similar tendency in the material immediately ventral to the early blastopore—that is, in the future yolk plug. This latter fact must be considered in our interpretation of the movement of material in the dorsal lip.

Figs. 27 and 28 represent an egg stained in the equatorial region of the early gastrula in such a manner that the stain extends in a meridional direction across the blastopore, considerably further than in the preceding figures. The result in later stages (Figs. 29 and 30) shows conclusively that the shifting of tissues toward the median line just below the blastopore is almost the mirrored image of that above the blastopore; the differences in the two levels at the ends of the blastopore are to be explained as due mainly to the process of convergence.

These results suggest a mechanical explanation of confluence. Does the material immediately above and below the blastopore merely follow the law of liquids flowing through an orifice, converging toward the point of least resistance? To test the matter I studied the currents produced in shallow water which was allowed to escape from a pan through a slit cut in the bottom. When the slit was made slightly wider at the middle than at the ends, the currents showed a tendency to converge, as shown in Fig. 31. In the blastopore of *Cryptobranchus*, the point of least resistance would naturally be at the center, where the invagination is deepest. But in comparing Fig. 31 with the preceding figures such as Figs. 25 and 29 one should remember that the stained areas do not represent the actual paths of moving particles—such paths are in reality much more oblique, as will be seen by comparing the original position of the stained areas. There is room for doubt if a mechanical explanation of confluence is really adequate to explain the extreme obliquity of the course taken by individual cells.

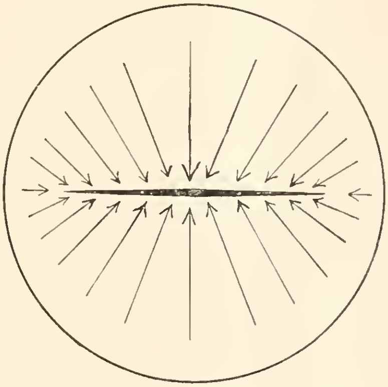
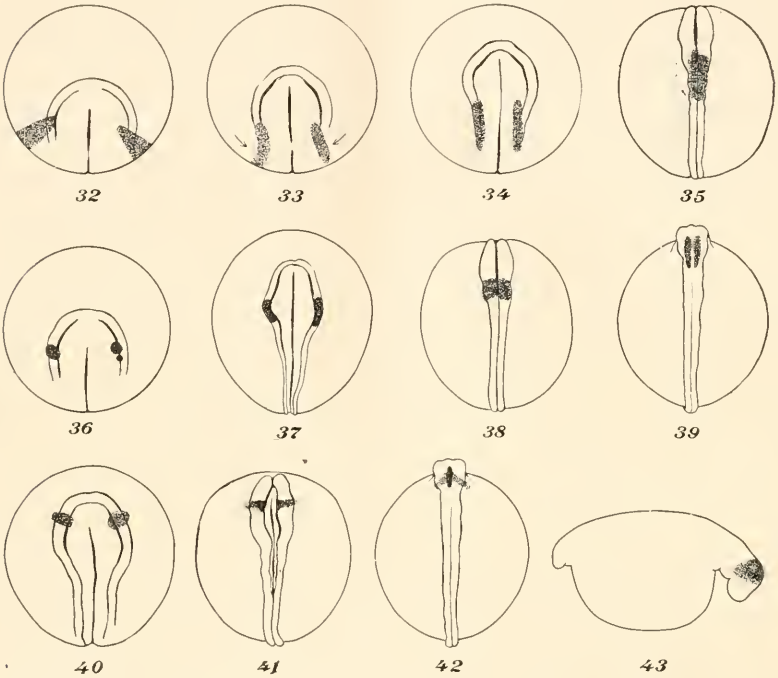


FIG. 31. Diagram showing the direction of the currents in water contained in a shallow vessel and allowed to escape through a slightly elliptical slit in the bottom.

C. The Movements of the Neural Folds.—We have spoken of the movement of the neural folds as a phase of concrescence in its widest sense; in this connection it may be worth while to examine into the character of this movement. Is it a mere wave-like undulation progressing from the lateral margins of the neural plate toward the median line, or is it a movement of translation whereby material is actually carried toward the median line? The study of transverse sections of a series of embryos inclines one to the latter view, and experiments prove conclusively that this view is correct. My results in general agree with and extend those of Goodale ('11) on *Spelerpes*.

Figs. 32 to 35 represent the history of an egg marked in the early neural groove stage. The distance through which material is brought from either side to the median line is remarkable, and in the absence of experimental demonstration would hardly be suspected. The neural folds, except their most anterior portion, are formed from material lying originally at least 90° apart, or one fourth the circumference of the egg. The forward movement



FIGS. 32 TO 43. Each horizontal row of figures represents the history of an individual egg of *Cryptobranchus allegheniensis.* The dotted areas represent marks made with Nile blue sulphate. Fig. 32, upper hemisphere of an egg in an early neural fold stage, sketched immediately after marking. Fig. 33, upper hemisphere of the same egg sketched a day later. Fig. 34, upper hemisphere of the same egg sketched a day later than the preceding. Fig. 35, dorsal aspect of the same embryo sketched a day later than the preceding. Fig. 36, upper hemisphere of an egg in an early neural fold stage, sketched immediately after marking. Fig. 37, dorsal aspect of the same embryo sketched two days later. Fig. 38, dorsal aspect of the same embryo sketched a day later than the preceding. Fig. 39, dorsal aspect of the same embryo sketched a day later than the preceding. Fig. 40, dorsal view of an embryo in the neural fold stage, sketched immediately after marking. Fig. 41, dorsal aspect of the same embryo, sketched a day later. Fig. 42, dorsal aspect of the same embryo sketched a day later than the preceding. Fig. 43, lateral aspect of the same embryo sketched three days later than the preceding. Four eggs were used for this experiment, with results so nearly identical that the history of any one of these eggs might be represented by Figs. 40 to 43.

of the stained areas is apparent rather than real, since it is due to the rotation of the entire egg on a horizontal axis.

Figs. 36 to 43 require no explanation other than that given in the legends.

The large number of experiments performed to demonstrate the movement of the neural folds gave remarkably uniform results, and clearly indicate that the movement is one of translation, and not a mere wave movement or undulation.

In distinction from the forms of concrescence more intimately related to gastrulation, the mode of concrescence of the neural folds and the gastral mesoderm might be distinguished as concrescence by apposition.

IV. Discussion.

In this paper the term concrescence has been employed in a wider sense than is usual in vertebrate embryology. One's views as to the scope of the term concrescence will naturally depend on his general theory concerning the origin of concrescence; on the other hand that theory is the most acceptable that gives a valid explanation of the largest number of facts, thus bringing them into a common category. The theoretical interpretation that appeals most strongly to the writer is the one stated by Wilson ('90). As the volume containing this contribution is scarce and almost inaccessible to many readers, a résumé of the most important points bearing on this problem is here given.

There are two views as to the significance of concrescence. According to the first view concrescence is a cœnogenetic phase of development; the embryo is temporarily bisected by the yolk, and the two parts afterwards brought together. Concrescence is thus a process of restoration, by which the two halves of the embryo, which have been mechanically separated by the yolk, are united. According to the second view concrescence has a far deeper meaning, and is palingenetic, though the accumulation of yolk may have modified its character. The second view is the one favored by Wilson, and requires further explanation.

The origin of concrescent growth is to be sought in the origin of bilaterality itself—an inquiry which leads us to the problem concerning the origin of bilateral animals from the cœlenterates, which by common consent are considered to have been their progenitors.

In the higher animals the gastrula stage is regarded as the embryonic representative of the radial, two-layered ancestral type; the blastopore is the representative of the single opening or so-called mouth of the cœlenterate.

Bilateral animals have arisen from radiate forms by the elongation of one of the transverse axes of the latter, the oral face becoming the ventral aspect, and the aboral face the dorsal aspect (this relation is reversed in the vertebrates). The mouth, meanwhile, shifted its position so as to lie near the anterior extremity of the new long axis, and the lateral portions, growing together more or less completely along the region formerly occupied by the mouth, gave rise to the process of concrescence in the ontogeny.

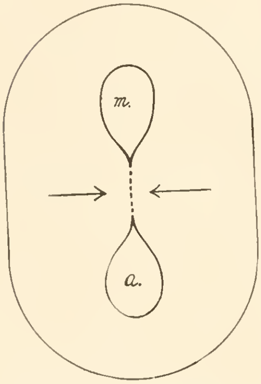


FIG. 44. Diagram showing the mode of blastopore closure in *Peripatus*.

What, then, was the origin of the anus in bilateral animals? In different embryos we find that the blastopore gave rise, sometimes to the mouth (through the persistence of the anterior part of the blastopore, as in the earthworm), sometimes to the anus (through the persistence of the posterior part of the blastopore, as in many vertebrates), and rarely to both (as in *Peripatus*, where the elongated blastopore closes in the middle). The only possible interpretation of these facts would seem to be that the blastopore originally gave rise to both mouth and anus. The case of *Peripatus* (Fig. 44) is then an interesting and apparently isolated remnant of the ancestral mode of development, or perhaps a reversion to it. It is interesting to note that in *Peripatus* the two halves of the body are quite well differentiated independently on the two sides of the blastopore; later, these two halves are brought together.

Concrescence is then a sequence of the closure of the blastopore, which primitively extended the whole length of the embryo, the anterior end forming the mouth. The separation of the two sides of the embryo, shown in the vertebrates most clearly in the early history of the mesoderm and the nervous system, was primitively caused by the blastopore itself. In the great majority of cases the original mode of closure of the blastopore has been secondarily modified, but the mesoderm and the neural cords or folds still follow the original mode of development, being laid down separately on either side of the region of the primitive blastopore, and growing together along its line of closure (concrescence by apposition). In cases where the region of the blastopore is occupied by a large mass of yolk, the process of concrescence is much modified and exaggerated; to this extent only, concrescence is to be regarded as a cœnogenetic character.

Taking up now, in the light of this theory, our special problem of concrescence in the vertebrates, we may note that there is no valid objection to the extension of the theory to cover the vertebrates on the ground that forms like *Peripatus* and the earthworm are not ancestral to the vertebrates; granting that these forms are probably far removed from the ancestral line of the vertebrates, we may still rely on the fact of common descent from the cœlenterates. Further, we may note that the ancestral mouth of the vertebrates has been lost, and a new one acquired; the anus represents typically the posterior remnant of the primitive blastopore. Wipe out the upper half of the figure showing blastopore closure in the embryo of *Peripatus* (Fig. 44), and we have left substantially the conditions found in the late gastrula of *Cryptobranchus*. If we give a phylogenetic interpretation to the confluence of materials in the dorsal lip of the amphiban blastopore, then this confluence is but a vestige of the more pronounced shifting of tissues that caused the constriction of the primitive blastopore. The observed confluence of materials in the dorsal lip of the blastopore is such as this theory of concrescence would lead us to expect. I have suggested a possible mechanical explanation, which seems not wholly adequate. The facts are not inconsistent with the palingenetic theory of concrescence.

The process which I have called convergence is necessitated by the large mass of yolk which greatly delays the completion of the process of gastrulation.

Like most phylogenetic speculations, the palingenetic theory of concrescence is weak in that it seems incapable of absolute demonstration. On the other hand the theory is useful in that it brings together in a single view a number of very important anatomical and embryological facts, putting them in an intelligible relation to one another. If we reject this theory, then we can scarcely consider the term concrescence to cover the behavior of the neural folds and the mesoderm in the vertebrates; concrescence becomes limited to events concerned with the present process of gastrulation, and probably applies only to the formation of the posterior end of the embryo. If we accept this theory, then concrescence has a broader and deeper meaning, and explains the most fundamental events in the formation of the embryo.

Perhaps the greatest gain that has come from the theory or theories of concrescence is that research has thereby been stimulated, with the result that we now have a much more accurate knowledge of the early stages of development of many forms than would otherwise be the case. The facts thus brought to light may be of greater value when considered from an altogether different angle, in the development of the various aspects of embryological science.

V. Summary.

By the method of vital staining the following facts concerning the formation of the embryo of *Cryptobranchus* were established: (1) A band of cells occupying the lateral and ventral parts of the equatorial region of the late blastula, during gastrulation comes to occupy the corresponding parts of the margin of the yolk plug, and converges on the site of the closing blastopore. (2) During gastrulation there is a confluence of material lying in the region of the dorsal lip of the blastopore: in connection with the process of overgrowth and in-turning of the dorsal lip of the blastopore, this material shifts from either side toward the median line. (3) The movement of the neural folds is a movement of translation, not a mere wave movement; the neural folds include material originally situated at least 90° apart, which is thus brought into apposition in the median line.

The bearing of these facts on the theory of concrescence is discussed.

Zoological Laboratory,
Michigan State Normal College,
Ypsilanti.

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THE BOTTLE-ANIMALCULE, FOLLICULINA; ŒCOLOGICAL NOTES.

E. A. ANDREWS.

The following observations upon the œcology of the marine protozoan *Folliculina*, known to some microscopists as the Bottle-animalcule, were made in connection with a study of its life history and method of forming its bottle-shaped case that will be published elsewhere.

In the summer of 1912, and again in 1913, vast numbers of these interesting protozoa and their cases were found on aquatic plants in the Severn River, which is a brackish side branch of the Chesapeake Bay.

The wide distribution of this highly specialized marine *Stentor* is indicated in the following brief outline of the history of our knowledge of it. Discovered by O. F. Müller in 1781 on the Danish coast, it was next studied on the Norwegian coast in 1858 by Claparde and Lachman and on the British coast by Strethill Wright; at Wismar by Stein in 1861; near Kiel by Möbius in 1865; again on the British coast by Saville Kent in 1870–1879; near Naples by Geza Entz in 1884 and in the same year by A. Gruber at Genoa.

Recently, 1910–1913, Carl Dons had described various *Folliculinas* from Norway, Spitzbergen and Iceland as well as the Adriatic, while Laachmann has found them in West Australia, Sumatra and the Antarctic.

The only records of its occurence in American waters is that of Leidy, who in 1859 found it at Newport, R.I., on the Atlantic coast of the United States while dredging with Col. Powel, and that of Ryder, who in 1880 described it as found in quantities on oyster shells from the west coast of the Chesapeake Bay.

In the sea *Folliculina* has been found living attached to various red and brown algæ and other plants as well as upon stones, the shells of molluscs, and annelids and the tests of hydroids, bryozoa and tunicates from shallow shore waters down to depths of four hundred meters. A few were taken on the surface of the water.

There are, however, records of its occurrence in fresh waters; thus Saville Kent in England described *Folliculina boltoni* from *Anacharis* and other plants as living in fresh water, and Barrett found it in the fresh waters of the Thames; while the form called *Ascobius lentus* by Henneguey in France found in flowing fresh water in the botanic garden at Montpelier on the leaves of *Aponogeton dystachium* seems to be a kind of *Folliculina*.

The *Folliculina* found in the Severn are no doubt *Folliculina ampulla* in the wide sense used by Stein and by Möbius and resemble most the forms described by Strethill Wright, but in the light of the revision of the group by Carl Dons the specific determination of these Severn forms is deferred till a more complete comprehension of their life history furnish a better basis than the mere form of secreted case the animal inhabits. Both *Folliculina* and *Parafolliculina* forms in the sense used by Dons occur here; but the character of nucleus and existence of micronucleus necessitates much modification in his classifications. It may well be that not only *Freia* and *Lagotia* but also *Ascobius* are synonyms of *Folluculina* and that many of the species are but phases in a protean life cycle.

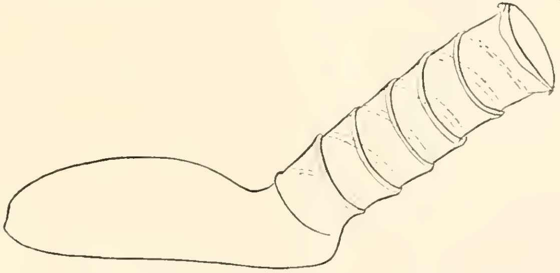

FIG. 1. Side view of case made by *Folliculina*. Camera lucida. × 233.

The most usual form of dwelling inhabited by *Folliculina* in the Severn is shown in Fig. 1 enlarged 233 diameters. For convenience of description it might be regarded as made up of a sac and a tube, both together constituting the case. The sac is the part of the case attached to some foreign object and the tube is the free part. The sac is wider from side to side than it is deep, since its attached face is flattened out against the support, while the free face or back is very convex. The posterior blind end is bluntly rounded and affords attachment for the foot end of the animal. The tube rises at about 45 degrees from the axis of the sac or the plane of attachment, and ends in a freely open mouth without valves but with a reflexed lip. As a rule about six turns of a spiral-ridge pass about the tube, but the number is not fixed. The spiral seen from the right side, Fig. 1, passes toward the top of the tube over the dorsal side. The nature and mode of formation of the case and the spiral of the tube will be described elsewhere.

Areal and Depth Distribution in the Severn.

First seen on the leaves of *Elodea* (*Anacharis*), the cases or bottles of these animals seemed to the eye mere dark lines $\frac{1}{2}$ mm. long that might be excrement of some small snail lying abundantly on the leaf amidst sediment.

But the microscope reveals marvelous greenish, transparent, bottle-like cases containing a clear, but blue-green animal that shyly withdraws till no longer disturbed and then may, from time to time protrude from the mouth of the bottle as a slender thread divided like a snake's tongue into two forks, which are held curved or else bent about and waved till all suddenly vanishes as a mere lump within the bottle. The animal might be compared to a miniature fork with long bent arms all made of glass but more pliant and elastic than rubber.

The cases with or without the contained animal were found on the leaves of several plants, chiefly *Elodea* and *Potamogeton* growing upon the bottom of this decidedly saline water, and also upon floating leaves and stems of these plants in the open river as well as on the sides of a boat and on wood. These plants form zones along the shores of most of the side branches of the river, known as "creeks"; the *Potamogeton* zone is nearer shore and grows in water one to two feet deep; the *Elodea* appears as a blue-green zone some twelve feet wide in water two to six feet deep, at high tide (the water falling one to two feet). *Folliculina* was taken from "Back Creek" below Annapolis up beyond the "White Sands," some nine miles, in side creeks on both shores.

The *Elodea* generally grows in a dense continuous plantation the crowded tops almost touching, so that in a square foot there is at least one plant with some fifty leaves on its upper four inches where the *Folliculina* tubes abound; on an average each leaf has five to ten tubes; many have much greater numbers. Allowing 250 tubes to the square foot, which is a minimum estimate for the more sparsely populated regions, and estimating the length of the *Elodea* zone as twenty miles and its width as only 4 feet, since the *Folliculina* are most abundant in water 3–4 feet deep and not in the upper and lower edges of the *Elodea* zone, we would have some one hundred million of these miniature dwellings along the Severn. They were found also in abundance at the head of Whitehall river which opens independently into the Chesapeake just to the north of the Severn, and as they are probably widely distributed along the branches of the Chesapeake we may regard *Folliculina* as a numerically important member of the summer fauna of this large body of salt water.

The following experiments throw light upon the distribution of the tubes of *Folliculina* in various depths of water. Late in July with water at 28° C. strips of smoothly sawed pine wood 35 mm. wide and 9 mm. thick were stuck upright in the mud in a row across the *Elodea* zone and left six days. On all strips in from two feet to five feet depth of water many tubes of *Folliculina* were attached, but they were absent from the bottom for some eight to twenty inches above the mud and from the top some nine inches below high tide line. The stick nearest shore in two feet water had a sparse population, perhaps 100 tubes to the square inch, while the sticks farther out showed in places as many as 400 per sq. inch. In these latter the tubes were often aggregated in clusters, one containing as many as 73 tubes, while in the more shallow water the tubes were scattered and more irregular in distribution. To see if the tubes would be made in depths beyond the *Elodea* zone the same strips of wood were tied together in a row, end to end, and anchored in the middle of the mouth of a creek where there was no plant life on the bottom, the water being 15 feet deep, and the *Elodea* zone several hundred feet distant. After a submergence of three days and four nights all the four sections of the compound strip were well set with new tubes (the wood was dried and sandpapered before this experiment). The lowermost section of wood reaching from the bottom up six feet showed tubes about twenty to the square inch with but few in small clusters, but the lowest two feet contained fewer and the bottom few inches, none. The second section five feet long had as many, if not more, tubes than the lowest section and more clusters, one containing as many as 20 tubes. The third section was three feet long and reached up above the surface of the water. The tubes on it were as on the section below it but became very sparse near the surface of the water. The fourth section floated out diagonally in the water but was only partly submerged and it contained but few tubes.

In brief the tubes had been made on the strips of wood from near the surface to within a few inches of the bottom, when there were no plants near from which the animals might have migrated.

It is thus evident that *Folliculina* occurs in greater depths than the zones of vegetation in these creeks, but normally it can find solids for attachment almost only within these zones, since the region is without stones or rocks and with teredo fauna that rapidly removes submerged woodwork.

Folliculina is thus forced into association in depths with *Elodea* and the few other flowering plants that follow the shore.

Seasonal Distribution.

These waters undergo great seasonal changes; in the high temperatures of summer the water is turbid with microscopic life, but more clear in winter.

The grosser life of the plant zones along shore also rises to a maximum in the summer and in the winter disappears down to the bottom of the water where only the roots and pieces of stems remain to revive in the following spring.

Folliculina is thus forced into seasonal periods of abundance and disappearance; but while it is abundant only with the growth of the plants it has a much shorter period than they, appearing in the summer after the plants are well started and vanishing long before the plant zone dies down.

In 1912 no live *Folliculina* could be found after September 8, though in aquaria they lived till the 27th and in a warm room till November 11, though in a faded and apparently starving state. In 1913 there was a sudden disappearance of most all live *Folliculina*, leaving chiefly empty tubes, after a sudden drop of temperature from 97° F. to 60° F. with rain. No live ones could be found after September 5 after cold rain, though found September 1 in a very few examples after much search.

By September 22 with the water at 83° F. and air at 80° F. no tubes even could be found except in places where the plant had not recently grown enough to shed the leaves to which the *Folliculina* tubes had been long attached.

Such empty tubes remaining upon old leaves that have not yet fallen off, illustrates one phase of the correlation between *Folliculina* and the growth of the plants.

Followed from spring onward the distribution of the living *Folliculina* is nicely adjusted to the growth of the plants, as will be seen from the following.

May 17, 1913, no *Folliculina* had yet appeared, but the *Elodea* zone denuded all winter had begun to be repopulated with new plants shooting up three inches high, apparently from old bits of stem buried in the mud. These plants were then perfectly clean and bright with no inhabitants save a few minute young snails. Some of the cells of the *Elodea*, however, were already dead here and there, apparently from some parasite; but this was not connected with *Folliculina* that at present was in some unknown state. The *Potamogeton* zone showed stalks eighteen inches high with some few flower buds far from the surface of the water.

By June first the *Potamogeton* had flowers near the surface and the *Elodea* was 12 to 15 inches high and no longer entirely clean but with the older lower leaves covered with a flocculent deposit and supporting many colonial vorticellids seen to the eye as contractile tufts on single stalks. Two weeks later, the *Elodea* was two feet high with flowers on stems four to six inches above leaves and some open flowers at surface. The *Potamogeton* now was three feet high with flowers at the surface of water. But all the leaves were covered with dirt and no *Folliculina* was found. On the 27th, *Folliculina* was found as black patches 1 to 2 mm. in diameter here and there on the leaves of *Elodea* in water two to six feet deep, otherwise the leaves were clean. These patches or colonies of *Folliculina* were only one to the leaf, either at the tip or at the edge of the upper surface. The origin of these first tubes was not determined. Aquaria that contained *Folliculina* in the fall still contained the empty tubes at this season but no revival of *Folliculina* was found in the aquaria even though the aquaria were kept all the summer.

Whether the *Folliculina* passes the winter in some form in the mud to develop when the conditions are right or whether it migrates into the river each season and becomes attached when the growth of the plants, the temperature and food conditions permit, is not known. The fauna of the Bay and river is markedly increased by migrants; not only are there vast shoals of jelly-fish (*Chrysaora* form of *Dactylometra*) and of ctenophores that come and go with menhaden and other fish but the plants that grow along the shores become the support for bacteria, diatoms, protozoa, anemones, polyzoa, tunicates and nudibranchs, that disappear in the fall and seem to have arrived as plankton and to have multiplied or grown in the temporary forcing house that this body of water forms in the summer. With the water gradually becoming opaque green from the multiplication of the microscopic biota and the temperature running up to 27° to 30° C. in July when the air is 28° to 29° C. *Folliculina* with many other organisms lives in ideal conditions for rapid multiplication, so that a few immigrants from the sea might produce the entire population that dies out at the oncoming of cool weather and the great changes in the gross and microscopic fauna and flora of the shores. Thus *Folliculina* and other marine animals become temporarily associated in a summer community dependent upon the zones of essentially fresh water plants, *Elodea* and *Potamogeton*.

The Chesapeake and its branches is a typical "drowned river" and we find here a tendency toward a gradual capture of the fresh-water biota by the incoming marine biota. Thus the marine blue-crab with many sea fish migrates over the line between the fresh and salt waters and at times becomes an important factor in the œcology of fresh and of marine organisms as well as of the dwellers on the transition line.

In the general process of capture of fresh by marine biota, may be included the temporary utilization of the fresh water plants, *Elodea*, *Potamogeton*, etc., by the summer immigrants from the sea. The swarms of *Folliculina* settling in the *Elodea* zone is but one illustration of the push from the sea inland.

The waters of the Severn support a thousand acres of natural oyster bars and are thus marine rather than freshwaters. The water in this river as published in 1912 by the Shell Fish Commissioners of Maryland varied from 1.0036, 1.0048 in July to 1.011 in December and 1.0096 in March; the gravity of the ocean being from 1.025 to 1.027. It should be emphasized, however, that each creek has some little spring or stream of fresh water entering its head as representative of the former headwaters of a side branch of the original fresh stream and where this water meets the saline tide waters there is an oscillation of conditions from the densities of high tides in dry seasons to the extreme freshness of low tides and in-pour of torrents of muddy water after rains. It is between these oscillation areas on the one hand and the more continuously saline depths on the other that the *Elodea* zone is most pronounced.

By July 4, *Folliculina* was very abundant over the leaves of *Elodea* in small scattered colonies of each a few individuals; by the 13th, exceedingly abundant on most all the leaves of the top two to four inches of *Elodea* in two to four feet depths in a zone 12 feet wide; they also formed a black area a few inches wide close to the surface of the water along the sides of a floating boat, twenty feet from the *Elodea* zone.

With the progressive growth of *Elodea* the upper leaves are the only ones upon which live *Folliculina* is found while the older leaves are thick-set with empty tubes. As the plant grows upward the shaded old leaves macerate and disappear but the tubes fall off to the bottom and may be got in great numbers from the mud as well as from the macerated dead leaves. In August, *Elodea* may be four feet high, but only the upper foot has leaves, and on these the dense settlements of *Folliculina* are generally a few inches from the top. Examination of the leaves shows the youngest Folliculinas on the uppermost or youngest leaves and so on down. Thus during the growing season there is a constant succession of new tubes formed above on the more recent leaves while lower down the old tubes are doomed to be dropped off from the macreated leaves to the bottom where they long resist decay. Both the gradual spread of *Folliculina* tubes from the first seen on the tips of the leaves over the entire expanse of upper leaves and the secondary migration from these leaves ever upward to other leaves as they grow out from the terminal buds might be accounted for on the hypothesis that the entire occurrence of *Folliculina* in these waters is the result of inoculation each spring with new individuals from outside the region and the rapid multiplication, spread and autumnal destruction, under seasonal changes of environment.

Sessile and Motile Forms.

Direct observations in aquaria showed that many of the above facts as to the attachment and occurrence of tubes were due to the existence and behavior of free swimming motile states of this animal. When brought into dishes of various kinds these motile forms soon swarm out from the tubes and construct tubes attached to the sides of the dish. Under the microscope, some of the *Folliculina* were seen to emerge from the tubes and to eventually swim free in the water. With the naked eye the free swimmers were followed in the aquarium till they became attached to its sides. When the *Elodea* was collected in the evening free swimmers were found the next morning and made new tubes during the day. In this way as many as four successive crops of newly attaching *Folliculina* were obtained in successive days from one collection of *Elodea*. The newly settled animals lived probably at least two weeks though the longest observation of individuals was nine days.

While most of the new swimmers came from old tubes on the *Elodea* there was good evidence that the new tubes formed in captivity gave rise to swimming forms. When collected early in the morning free forms were found before noon and new sessile forms were made in the afternoon. While the temperature seemed to influence the times, in general no swimmers were liberated in the night but began about seven or eight in the morning and swam two to several hours; about noon the sessile form was begun with the making of cases which were completed in from four to eight hours; in the evening one to several hours, often, were taken to perfect the organization of the sessile form in its new case.

Within the twenty-four hours the entire cycle from sessile to new sessile form was completed, and in extreme cases, only six hours were necessary.

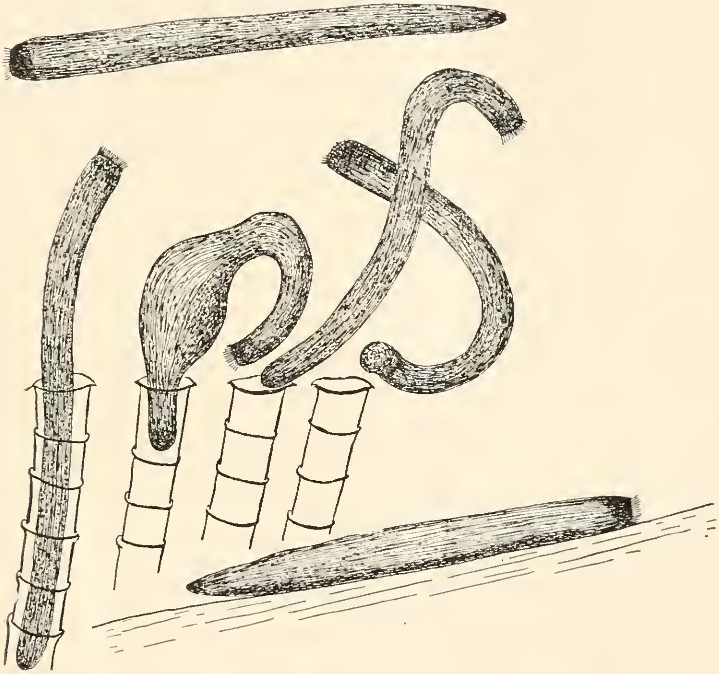

FIG. 2. Sketch of motile form emerging from tube. Four successive stages of one individual in row from left to right with same individual above swimming free and later, below, gliding over surface of leaf.

In nature then it is probable that the *Folliculina* on leaves of *Elodea* give off swarms of motile forms that find the new upper leaves, as well as floating leaves and objects in the deep waters also as shown in the above experiments, and quickly settle down to remain in new tubes till these leaves in turn become unfavorable from accumulations of dirt and from shading by overhanging leaves. Then there is new migration upward and so on as long as food, temperature and other conditions allow.

Failure to collect such motile forms in the open by dragging nets over and through the *Elodea* was in part due to the lateness of the season, August 19–20, and in part to the difficulty of retaining ciliated cylinders one tenth to one twentieth of a millimeter in diameter in a bolting cloth of one tenth millimeter mesh.

The motile forms must be very abundant at times in all these waters and through the summer floating branches of *Potamogeton* and of *Elodea* taken in the middle of the river are more thickly covered with tubes than those growing along shore, as if they had been colonized while floating. Far swimming motile forms carried by the currents may be the first settlers in the spring that so rapidly take possession of all the *Elodea* zones. Such motile forms must be a considerable addition to the plankton and may fall a prey to the schools of young menhaden that frequent the river and its branches, feeding over and near the *Elodea* zone as well as farther from shore.

That the sessile forms have some enemies is seen in the utilization of their cases by the larvæ of aquatic insects that bite them off and from them construct sinuous tubes in which they lie stretched out over the surface of the leaves, with green contents in their digestive tracts strongly suggesting that they not only destroy the cases of *Folliculina* but eat the contained animals; and one larva was seen to bite off and chew up a *Folliculina* recently settled on filter paper.

The distribution of the *Folliculina* cases over the leaves being due to the behavior of the motile forms, two questions call for answer; why are the upper leaves selected and why are the cases placed in groups or aggregates? The following observations on the actions of the motile forms aid in answering these questions.

Behavior of Motile Forms.

The motile forms seen emerging from tubes, Fig. 2, are long cylinders abruptly truncated at the anterior end and bluntly pointed at the posterior end. They change form by local contractions, bending the part outside the tube in various directions and swelling and contracting in diameter in some regions at expense of others. The figure represents one in successive poses from left to right, finally leaving the tube. After some minutes, of thus, as it were, feeling about, the *Folliculina* swims away from the tube, as in the upper part of Fig. 1 and then resembles a spirostomum. It may sooner or later settle upon leaf or stem and glide about, as in the bottom of Fig. 1, resembling then a planarian in motion. While very often an elongated cylinder it changes in swimming or gliding to various shapes, bending and contracting, may often assume a bottle form from the swelling of the posterior end, become flattened or take the form of a spheroid. The body is generally dark blue-green with the anterior part frequently much darker than the rest since a short region back of the truncated end is sharply set off as almost black and very opaque. The posterior end is frequently dark also.

The form assumed is often that of some species of *Stentor* in motion, and study of compressed living as well as sectioned material shows that they have the organization of *Stentor cœruleus* as described by Schroeder and verified by me on the same form for comparison; but they present certain marked simplifications at the anterior end and a complexity at the posterior end that will be elsewhere described.

These swimming forms show a great diversity in size as well as in form and in color density. Often they measure about one half millimeter in length.

The locomotion seems due to the longitudinal rows of cilia that cover the body and not to the abbreviated spiral of membranellæ at the anterior end. They swim forward with rotation, the right side coming up and over to the left, probably four to five times a second. The speed is two to four millimeters per second, but it changes. After long continuous stretches of motion it may advance, with interruption in jerks, as if the cilia stopped and resumed. Frequently the direction is changed and the phenomena of reversal and advance in another direction on approaching the edge of the water seem like those of *Paramœcium*. While the trajectory appears straight, often, it may again be a long sinuous curve and often a spiral. After stopping the animal may turn off and progress at an angle with its former course, as above stated, or it may move backwards nearly on its trail. It may reverse, spin on its transverse axis and then resume the old direction.

In a very small drop of water the *Folliculina* may go back and forth again and again like a trolley car, reversing at each end of its short trip. One individual with a fixed black spot on the anterior end was seen to go forward and backward and to spin about its transverse axis without revolving about its long axis.

Responses to Light.

It is very striking to see the completeness with which light determines the distribution of the swimming *Folliculina* in aquaria. If in an opaque dish the tubes are all made on the side most illuminated, towards the window in a room, or if in a square glass vessel all tend to crowd to the angle nearest the strongest light window. Watching the swimmers we see that they go rapidly toward the light and only turn back upon striking an obstacle such as the side of the dish when they may swim away for a distance soon to return and eventually to remain nearest to the light. The new tubes are then built along the side toward the light and most all are very near or at the surface of the water unless some irregular reflection determines the formation of two bands, one near the surface and one deeper down, but while the densest crowd is close to the surface, tubes are made an inch and a half below the surface in a scattered arrangement. New tubes formed on strips of paper and glass slides at the side of the dish were removable for study and preservation. When the aquarium is placed on the floor and lighted largely from above, many tubes are formed on the surface of the water, either on floating objects as cover glasses, pieces of paper, leaves, etc., or merely on the surface film. In this way new tubes with the contained animals were obtained free from all solid substratum and excellent for sections and other manipulation.

By placing an opaque paper with central hole over the dish containing the *Elodea* and old *Folliculina* the new formed swimmers were concentrated to the center of the dish and could be taken out with the pipette or later taken up as floating colonies away from the sides of the vessel and in a limited area.

While most all of the swimmers collect toward the light some may form tubes on the sides of the vessel away from the light, but these are much in the minority. In a watch-glass the swimmers go at once to the side whence the light comes.

Responses to Solids.

On emerging from the old tube the swimmer may not at once swim toward the light but glide along on the leaves of *Elodea;* such rapidly gliding *Folliculina* suggest planarian locomotion; they seem investigating the surface by bending in and about amidst diatoms and detritus and crawling over complex surfaces as if attracted by contact. After swimming to the sides of a vessel the *Folliculina* is markedly affected by contact, often at once ceasing to swim and beginning to glide along the surface of whatever nature it may be; but this adjustment to the surface does not necessarily dominate for long, as the animal may suddenly swim free again for a short distance away from the surface to return again. In this way it is a gradual process of coming and going that finally results in the arrest of all motion on the surface of future attachment of the new cases. When arrested by a surface the animal abruptly changes form, frequently becoming spherical or bottle-shaped. In some cases the body is markedly flattened out against the surface and the form may be that of a pocket flask some two times as wide as thick. In gliding as in swimming, *Folliculina* may advance in jerks, or abrupt changes at very short intervals.

While the free swimmer comes to rest and adheres to the surfaces of leaves, paper, glass and porcelain as well as rough wood there are some surfaces that are less available as indicated by the few tubes formed on the resinous parts of the pine strips above réferred to, and by the failure of the following experiment.

August 5, in a region where *Elodea* was nearly black with tubes of *Folliculina* and many were also present upon *Potamogeton,* plates of wax "foundation" as used by apiarists were suspended in wooden frames so as to float just above the *Elodea* in five to six feet of water. In six days but three or four very young tubes were found on both surfaces of the two sheets of wax, though the tubes were scattered over the narrow wood frames. Ten days later the tubes were more numerous on the wood, but only a dozen were on the wax.

Though these experiments were tried too late in the season to get a marked attachment of free swimmers yet it shows that the small area of wood was more effective than the greatly larger area of wax, though the latter presented many facets and edges. To this evidence of discrimination in the surfaces settled on should be added the fact that the tubes were not made on the lowermost inches of strips of wood, even when there was no plant present to shade the bottom (as above described) and few tubes were attached to the more resinous parts of the wood, so that the swimming *Folliculina* would seem to react to various stimuli.

Again in the experiments with strips of wood the tubes fastened to the wood both in shallow and in deep water were noticeably orientated with the mouth of the tube upward toward the surface and the tubes were prevailingly situated lengthwise in the furrows of the wood caused by the saw and grain.

On the stems of *Potamogeton* also the tubes stand lengthwise and generally with the mouths upward. On leaves the tubes are generally more abundant upon the upper than the lower surfaces and often are crowded toward the edges.

The above response to light would lead to the successive colonization of upper leaves in the *Elodea* zone during the season and would explain the common orientation of the tubes with mouth upward.

The responses to solids would lead to the rapid utilization of most solids as basis for attachment of tubes. But evidently some other factors must be concerned in the choice of certain surfaces, or refusal of some, and in the selection of certain sites, as grooves and especially the crowding into aggregates as if aware of one another's existence.

Aggregation.

Though isolated cases or tubes of *Folliculina* are common on leaves as well as upon material experimentally supplied in the open and in the laboratory for the attachment of the swimming forms, yet it is very noticeable that the great majority of *Folliculina* tubes occur in groups as if arising as colonies from budding or in some peculiar way associated with one another.

While such aggregates are well shown in nature on leaves of *Potamogeton* and *Elodea* they can be better illustrated from the groups formed in aquaria on paper, glass and the surface film of the water.

In Fig. 3 is represented a fine example of an aggregate formed in the following way: the *Elodea* from the river was placed in an open dish with opaque sides and motile *Folliculinas* swarmed out, moved toward the light above and came to rest upon floating objects. In this particular case a floating coverglass of 18 mm. side received this one aggregate of *Folliculinas* and no other individuals at all. That is for some reason all the *Folliculinas* that came to this area of 324 sq. mm. and settled down, made tubes in one region of only about one sq. mm. very near the center

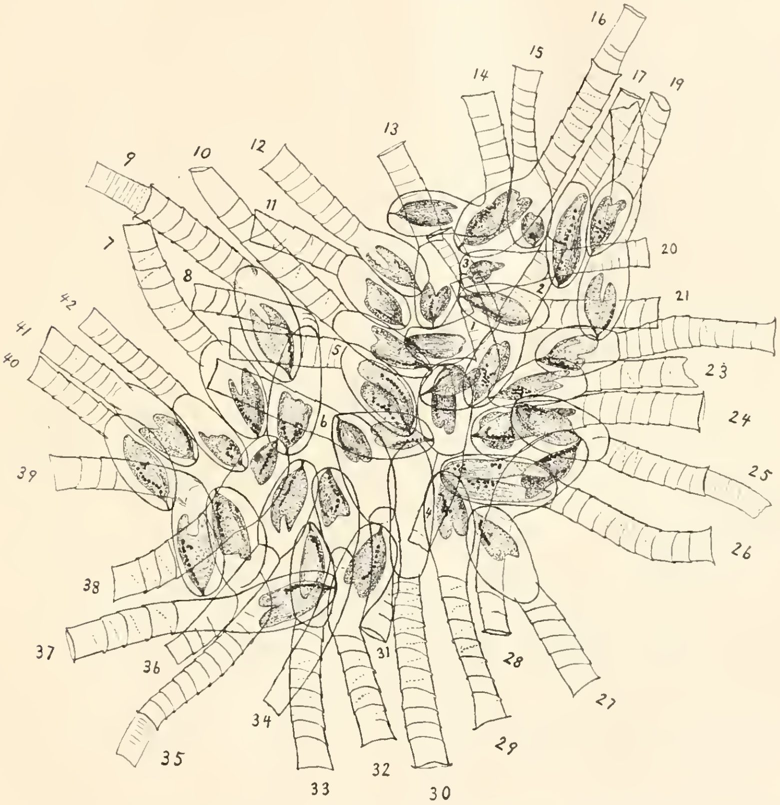

FIG. 3. View from above of a group of 42 that are attached to a floating cover glass with the sacs fastened toward the observer and the tubes reaching down away from him. With the exception of 1, 2, 4, 5, 6, all radiate out around the periphery of the group and most are attached in one plane. Camera lucida from preserved specimens. × 44.

of the entire glass. In other cases such settlements are made at the edges, or here and there over the glass. Aggregation into groups is the general rule.

It will be noted that the 42 individuals of this aggregate are most all in one plane, spread out over the glass and attached to the glass so that the flat side of the sac, see Fig. 1, is fast to the glass while the tube of the case rises up away from the glass at an angle of nearly 45 degrees, Fig. 1. Fig. 3 was drawn with the camera from the upper side of the cover glass to which the cases were attached below, so that the tubes pass downward from the level of the sacs. While most of the sacs stand side by side in one plane, some overlap and some are fastened deeper down in the water on top of the general stratum of sacs. It is noteworthy that the tubes of all the cases radiate outward, with few exceptions.

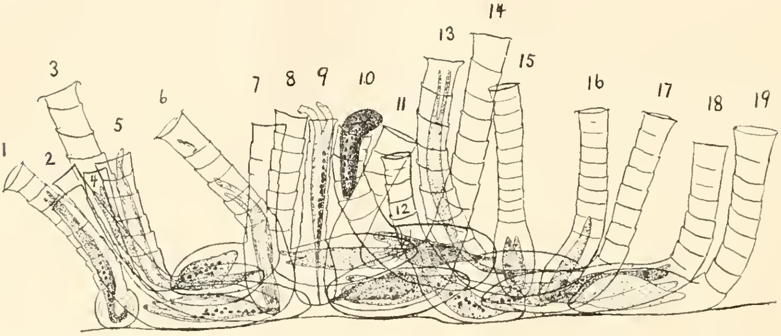

Fig. 4. Side view of a group of nineteen individual *Folliculinas* that made these cases on the edge of a cover glass floating on water. While most of the sacs stand in a row some are behind others and some are across the general trend. A few lie piled upon the others. One, No. 10, is in process of escape from the tube. Camera ucida, preserved material. × 55.

Most of these forty-two are generally alike in size and structure of cases, and with the spirals in the same direction. The animals themselves are but poorly shown, since they were distorted by treatment with too much cocaine in attempting anesthesia; but they show the moniliform nuclei and the stalks of attachment to the bases of the sacs.

Exceptional tubes similar to those of Wrights' specimens are shown in Nos. 9, 16, 25, and 35 in which a second story has been built out from the old mouth of the tube, and this added extension has but imperfect spiral ridges.

Another aggregate of only nineteen individuals is shown in Fig. 4, as seen from the side. This was formed in the same way but on the edge of another floating cover glass. The continuous line they are attached to is the continuous film they secreted and which was pulled off from the glass with all the cases in making this preparation. Though the thickness of the cover glass was but 150 μ. some sacs are placed back of one another on the end of the glass and so are partly hid by those in front, in the sketch.

Others, however, as 6 and 8, are not fast to the glass but are built up on top of other cases. Here again the cases are densely crowded together with very little space not occupied and the tubes radiate outward from the group as a whole, though in this case the group has the form of a linear aggregate.

In this preparation the animals are better preserved; No. 9 is about to expand outside of its tube; No. 13 is much elongated while in No. 10 is one caught in the act of escaping from its tube to swim away as the motile form without the characteristic long arms of the tube dwellers.

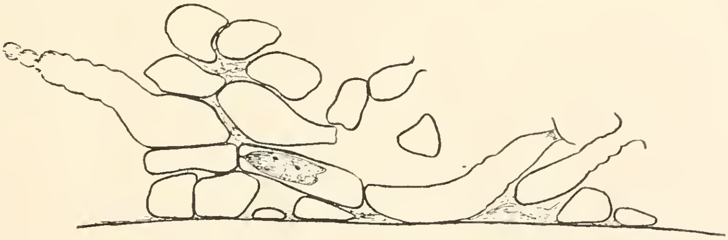

FIG. 5. Section, 6 μ thick, of a group formed on surface of water, to show that not all are on one plane but some are piled on top of others. All are held together by or attached to, a common secretion. Camera lucida × 66.

That the cases of *Folliculina* are not only made in contact with the foreign substance but also in contact with other tubes and above the general level is shown in actual sections of such aggregates. Thus in the 6 μ section, Fig. 5, several cases are seen piled up on top of others that rest on the common basis which was a pellicle formed by the *Folliculina* on the surface of the water. Between some of these cases not in close contact there is a mass of coagulum that binds them all together to some extent.

The material binding the cases together, seen as a film in Figs. 4 and 5, is so tenuous and transparent as to easily escape notice, but when the aggregates form on the surface of the water it is this common basis that enables one to pick up the aggregates as one mass and when the leaves of *Elodea* die and macerate, it is easy to obtain innumerable aggregates falling off but each firmly bound into one unit by the film that underlies all the cases.

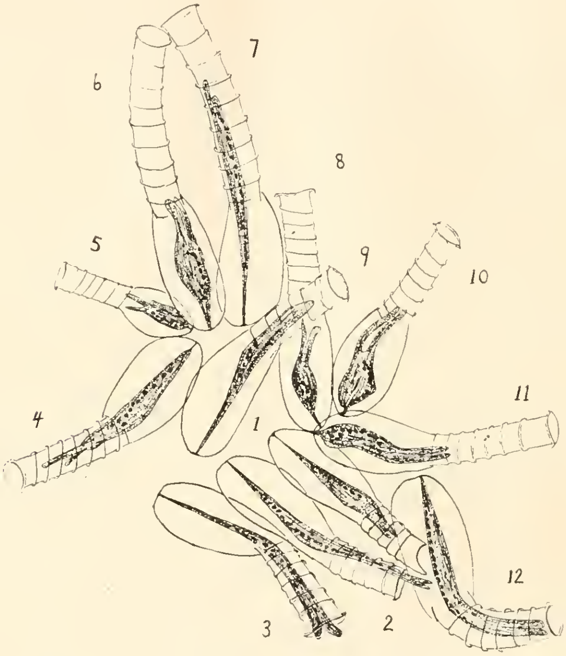

FIG. 6. View from above of a small group of twelve formed on surface of water. Camera lucida × 55.

Even such a loose aggregate as that in Fig. 6, formed on the surface of water is really bound into one mass that may hold together through all the processes necessary for section cutting, though in face view the film is not seen. This last figure well illustrates the tendency of the *Folliculina* to build tubes in a radiating group and to fill in most of the intervals in an irregular way.

Observations upon free swimmers and those making cases throw light upon the above groupings of the cases. The factor of importance in addition to the responses to light and to foreign objects seems to be the secretions put out by the free swimmers.

That the swimming forms may put out considerable amounts of invisible and adhesive secretions was shown in various indirect ways. Thus when india ink is rubbed up with the water in which the *Folliculina* swims, the presence of a secretion following the swimmer is evident. On the addition of granular carmine, swimmers were seen followed by long strings of granules and some included diatoms trailing behind the *Folliculina* four times its greatest length.

Again when the swimmers press upward into drops of water spreading up onto the edges of a procelain dish they get into water films of great thinness and here there seems a distinct film of bluish green material which remains on the porcelain after the *Folliculinas* have been removed.

That this secreted substance must be very adhesive is to be gathered from several cases in which a motile form came into contact with the larva of some aquatic insect, apparently dipterous. These larvæ are relatively large so that the *Folliculina* glides up and down and around the larva, whose diameter is much greater than the length of the *Folliculina*. The tendency of the *Folliculina* to remain attached to the surface of the larva while gliding all over it was most pronounced and its adhesion to it very strong, for the insect violently coiled in figures of eight and struggled with its legs without dislodging the *Folliculina*. When after many minutes the *Folliculina* left the insect a strand of slime was seen stretched between; presently when the insect came near but not into contact with the *Folliculina* they stuck together and the gliding over the surface was resumed.

Some motile forms about to make cases in the film of water rising on the edge of a watch glass were bound together by fine threads of slightly greenish material. Observations of motile forms about to settle down and secrete the case, shows that they come to rest gradually, moving in ever smaller areas and often, as it were, skating about on the posterior end and then flattening the whole body against the substratum. One may become attached by the posterior end and feel about like a leech and later swim off in a short arc to settle again. When carmine grains were added to the water it was evident that the animal was attached by the posterior end to an invisible film or raft of secretion that formed a pellicle over the surface of the water and extended out irregularly in all directions to a distance of one to four times the length of the *Folliculina*.

These thin pellicles that moved on the water with the contractions of the *Folliculina* were probably made by the individual animals from secretions of an adhesive nature and if other *Folliculina* had come near they might have had their motions influenced by this secretion so that they would have tended to settle down in the same neighborhood and so on till many individuals might become crowded together in one region and have all made their cases together as seen in Figs. 6, 3, and 4.

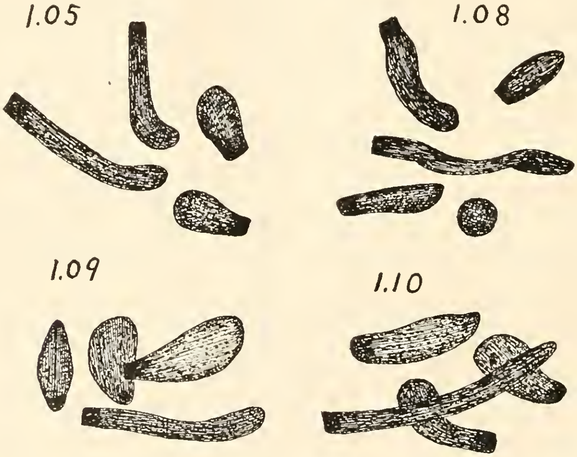

Fig. 7. Sketch of four successive groupings of motile forms that are settling down to form cases. Drawn at intervals of a few minutes from 1.05 to 1.10 p. m.

That several active *Folliculina* may be influenced to act together in one region is shown by the observations illustrated in Fig. 7. Here four motile forms attracted to the same spot remained for a long time with rapid changes of form and position within the group but did not move away from one another's company. Sketched at 1.05 o'clock, the four were radiating away from one another, at 1.08 a fifth animal approached but soon left the group; at 1.09, three were markedly flattened as indicated by the apparent increase in size, while at 1.10, they had altered positions and shapes. Such oscillations as shown in these five minutes might ultimately result in adjustments of many as in Fig. 6. The common factor that restricts the motions to small areas may well be the secreted film that limits motions by its adhesiveness. The secretion of the first that put out material in the substratum might delay the swimming of another that came near by, lessening the effectiveness of its cilia, mechanically; but other more complex reactions of the second to the secretions of the first may be imagined.

While the secretions made by those settling down on foreign bodies may somehow determine that other *Folliculina* crowd to the same region, special observations and experiments are needed to determine why the cases have the radial arrangement shown in Figs. 3, 4, 6. It is noteworthy that the cases lie either side by side as 1, 2, 3, Fig. 6, or else are so placed that their long axes radiate roughly from some central region. In the prevailing radial arrangements the anterior ends of individuals do not face one another but face away from one another at opposite poles of the radii of the group. The behavior of the motile forms indicated in Fig. 7, suggests a long series of trials or changes of position leading up to the final position assumed when the cases are made, but these animals did not make their cases for several hours and that seems so abnormal that one cannot rely on these specimens as showing the usual mode of settling down though we make use of them as evidence that they are held in one region by something which may well be the secretions.

Possibly careful study would show that the currents set up by the cilia would tend to passively drive the animals into the radial position when the posterior end of each is held somewhat passive by the abundant secretion at that end; but on the other hand some of the groupings, and especially the arrangements of *Folliculinas* that settle down on top of a group that has already made cases suggest that each animal is capable of responding to the presence of others in some complex way, so as to avoid facing another, and so as to fit itself into vacancies amidst a group or to lie parallel to others.

There remain other facts that must wait further observations of elucidation. Why do individuals prevailingly lie along in grooves of the wood? Why do they collect in groups near, but not at the edges of leaves? Why in such cases as Fig. 4, are they confined to the narrow edges of the glass and not extended over the lower face cf the glass as well? Why do they not settle on wax or on resinous wood?

Possible many of these facts result simply from the reversing movements on receiving a stimulus; an edge to a groove or an edge to the leaf, or the contact with another animal or with its currents may bring about the same inability to advance over the boundary.

Possible, again, there may be some such problems here as seem to exist in the earthworm in responding to angles and edges. The selection of the middle of the cover glass in Fig. 3, and the settlements along the top of leaves near the edges may involve some resultant of spaces travelled over after stimulation at edges.

While, in a general way, the action of the motile forms toward light, surfaces and in their own secretions may suggest how the cases come to be placed as they are in nature, yet there is probability that the phenomena are much more complex than as yet made out.

SUMMARY.

1. This protozoan occurs in the brackish waters of branches of the Chesapeake Bay in temporary association with the fresh water plants *Elodea* and *Potamogeton*.

2. Its adjustment to the growth of these plants is brought about by the migrations of motile forms that escape from the tubes of the sessile forms.

3. These motile forms respond to light and to solids, and these actions seem to keep them ever settling upon newer parts of the plants during the season.

4. The motile forms add materially to the plankton during the summer.

5. *Folliculina* appears in the early summer and disappears before the plants die down in the autumn, forming part of a

temporary community in which marine forms depend upon the fresh water plants for attachment and existence above the bottom. Presumably these animals migrate in from the salt water and die out every year.

6. The peculiar groupings of *Folliculina* suggesting some common bond may be partly explained as due to the secretions they put forth when about to build their cases.

7. The anatomy of the motile and sessile forms and the mode of formation of the case will be considered elsewhere.

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ASEXUAL BREEDING AND PREVENTION OF SENESCENCE IN PLANARIA VELATA.

C. M. CHILD.

In an earlier paper[1] the asexual life-cycle of Planaria was described and it was shown that senescence leads to fragmentation of the larger part of the body, the fragments encysting and undergoing, like other isolated pieces, reconstitution into small whole animals which when they emerge from the cysts are physiologically young and capable of growth and repetition of the life-history. So far as known this is the only method of reproduction in the localities about Chicago where these worms are found, sexual reproduction never having been seen during some twelve years of observation.

I. Asexual Breeding.

In order to determine whether repeated asexual reproduction was possible without senescence of the stock the asexual breeding of the animals was begun in the spring of 1911 with a stock of animals collected from a temporary ditch in which the species was abundant. In the attempt to find suitable foods the stock was given varied food including lean beef, liver, kidney, earthworm, fresh water mussel, crayfish, etc. In course of time it was found that the life-cycle could be considerably modified by different kinds of food and the results of the feeding experiments will be presented in another paper. After several months beef liver was found to be the most satisfactory food among those tested and has been the sole food of the stock since the early months of the experiment.

The worms of the stock collected in April, 1911, reached the end of their growth period, ceased to eat and began to fragment two to three weeks later and the cysts being kept in an abundant supply of fresh water during the summer, the young animals emerged during September and October and after feeding grew rapidly and in about a month attained full size and began to fragment and encyst again. The worms of the third generation emerged from the cysts in three to four weeks and again attained full size in a little over a month and fragmentation and encystment began for the third time.

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[1] "The Asexual Cycle of *Planaria velata* in Relation to Senescence and Rejuvenescence," Biol. Bull., Vol. XXV., No. 3, 1913.

Up to the present time this cycle has continued to repeat itself more or less rapidly according to the frequency of feeding. During the last year the animals have been fed only three times a week instead of every day and growth has consequently been less rapid. For two months in the summer of 1913 the stock was placed in the refrigerator at a temperature of 8–10° C. in order to avoid possible loss from high temperature, and during one month of this time the animals were not fed. This procedure had, however, no other effect than to bring about fragmentation in a few individuals and a slight degree of reduction from starvation. The few fragments were discarded and in September the animals were brought back to room temperature, feeding was resumed and growth began again.

In this manner the stock has been carried through thirteen asexual generations in less than three years. At present (March, 1914) the thirteenth generation is approaching fragmentation and encystment.

The stock shows no indication of loss of vigor. The animals which emerge from the cysts in each generation show a high rate of metabolism and are physiologically young and undergo senescence in each generation in the same way as the animals in nature. During the period of breeding there has been no indication of the development of sexual organs so far as could be determined by external examination. Certainly no genital openings have ever developed and no eggs have been laid.

As compared with the almost four thousand asexual generations of *Paramecium* bred by Woodruff the number of generations attained by this stock is small but the fact that a metazoan species is capable of passing through twelve asexual generations without any indications of sexual reproduction is of interest.

Moreover, it is not because the animals do not become old that they are capable of continued asexual reproduction. It was shown in the paper on the asexual cycle of this species referred to above that senescence is manifestly associated with growth in each generation and that the occurrence of asexual reproduction in this species is a result of the decrease in rate of metabolism which occurs in the course of senescence. In that paper it was also shown that asexual reproduction, which is essentially a process of isolation of pieces and their reconstitution into whole small individuals, brings about rejuvenescence as a result of the reorganization and reduction which occur in the process of reconstitution. Evidently the animals undergo a regression to a comparatively early stage of development with each reconstitution and it is also evident that the degree of rejuvenescence in each reproduction is on the average the same, for the stock does not as yet show any indication of a progressive senescence from generation to generation.

Whether gradual, progressive senescence of the stock is entirely eliminated by the process of reproduction or will sooner or later become apparent with continued asexual breeding can of course be determined only by further breeding of the stock and it is the writer's intention to continue the experiment as long as seems necessary. But the apparent absence of sexual reproduction in this species under natural conditions, to which attention was called in the earlier paper, constitutes strong evidence for the conclusion that the species is able to maintain itself indefinitely by asexual reproduction alone. Since it is demonstrated that asexual reproduction brings about rejuvenescence in this species as well as in *Planaria dorotocephala* there is no apparent reason why asexual reproduction should not continue indefinitely without sensecence of the stock or race. All that is necessary for the realization of this possibility is that the degree of rejuvenescence in each generation should be on the average the same and that is apparently the case.

II. Rejuvenescence by Starvation.

Planaria velata like *P. dorotocephala* and other species of *Planaria* undergoes reduction in size when starved and this reduction may be continued until the animal is but a small fraction of its original size. The reduction in size is of course due to the fact that the animal uses up its own tissues as a source of energy and since it contains no skeleton which takes little or no part in reduction a very great decrease in size may occur before death.

In a recent paper[1] it was shown that in *Planaria dorotocephala* the susceptibility of the animals and also the rate of CO_2 production increases as the animals undergo reduction from starvation. These changes certainly indicate an increase in rate of metabolism during starvation and reduction. The only difference between the animals reduced in size by a long period of starvation and young growing animals is that the starved animals possess almost no capacity for acclimation to low concentrations of KCN, alcohol, etc., while the young growing animals possess a high capacity for acclimation. When the starved animals are again fed this difference disappears almost at once and they are in all respects physiologically young and are capable of renewed growth and of repeating the life cycle.

In *Planaria velata* the same increase in susceptibility occurs during starvation as in *P. dorotocephala*, the reduced animals show almost as high a susceptibility as young growing animals of the same size and when feeding is resumed this species is also capable of renewed growth and of once more going through the life cycle. The susceptibility determinations give essentially the same results as in starved individuals of *P. dorotocephala*. Estimations of CO_2 production and determinations of capacity for acclimation have not been made for starved individuals of *P. velata* because it seemed unnecessary in view of the other facts.

There is then no doubt that starvation and reduction bring about rejuvenescence in *P. velata* as in *P. dorotocephala*. The large old worms with low susceptibility before starvation are in essentially the same physiological condition after starvation and reduction followed by renewed feeding as young growing animals of the same size and are capable of repeating the life history from the stage at which feeding is resumed.

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[1] Child, C. M., "Starvation Rejuvenescence and Acclimation in *Planaria dorotocephala*," *Arch. f. Entwickelungsmech.*, XXXVIII, 3, 1914.

III. Inhibition of Senescence by Partial Starvation.

If complete starvation and the resulting reduction bring about rejuvenescence it should be possible by feeding animals enough to prevent reduction but not enough to permit growth to keep them indefinitely in practically the same physiological condition and so to prevent senescence.

In 1911 a part of the stock used for asexual breeding was isolated in the second asexual generation after collection and the attempt was made to feed this stock only enough to maintain the worms at approximately the same size. During the early part of this experiment too much food was given and a few of the animals underwent partial fragmentation and encystment. All such individuals were removed from the stock and the experiment was continued with the remainder, these being completely starved for several weeks after fragmentations occurred in the stock in order to reduce their size and bring them back into a physiologically younger condition in which fragmentation would not occur. Since the early part of the experiment, the food has been somewhat further reduced in quantity and no further fragmentations have occurred. The stock was small at the beginning consisting of some forty worms. Some of these were lost by the early fragmentations and since that time animals have occasionally crept out of the water and dried upon the sides of the dish and others have been lost by being removed on the pieces of food or have been accidentally poured out in changing water but there have been no deaths or losses from other than these accidental causes. The stock now consists of five animals. As regards feeding the procedure finally adopted and still adhered to is to feed two or three times at intervals of two days, and as soon as the animals begin to increase in size to stop feeding for two or three weeks or until they are reduced to their former size. In this way the animals have been kept during most of the two years between four and seven millimeters in length. Whenever individuals of the stock show more rapid growth or reduction than the others they are isolated and fed or starved until they are of the same size as the others when they are again returned to the stock. During most of the time the stock has been fed with pieces of earthworm, because experience has shown that with this food the animals in general attain a larger size before ceasing to feed and undergoing fragmentation than when fed with liver. But when earthworm is given in sufficient quantities senescence occurs, though its course is somewhat different from that of senescence with liver as food. The effects of different foods on the course of the life cycle will be discussed at another time.

During the months of July, August and September of 1913 the stock was kept in a refrigerator at a temperature of about 10° C. in order to avoid the danger of encystment from high temperature, and feeding during this period was of course less frequent since the animals were less active and required less food to maintain a constant size. During September they were not fed at all and underwent reduction to a somewhat greater extent than usual in the starvation periods. At the beginning of October they were brought back to room temperature and since that time the feeding has been continued as before.

The five worms which now make up the stock are in the same generation as they were two years ago and of somewhat smaller size, about five millimeters, than at the beginning of the experiment. They are active, behave like young animals, react very strongly to food and appear in every respect to be as young physiologically as growing worms of the same size. Unfortunately the small size of the stock has not permitted determinations of susceptibility at intervals, but judging from the activity and appearance of the animals their susceptibility would be that of young animals. While these animals have remained in the same generation during more than two years and are to all appearances as young physiologically as at the beginning, in fact somewhat younger, since they are kept at a smaller size than when first isolated as a stock, the other portion of the same generation which was used for asexual breeding has in the same length of time passed through twelve asexual generations with the cycle of high susceptibility, growth with decreasing susceptibility, cessation of feeding, fragmentation, encystment, reconstitution in the cysts and emergence in each generation. In these partially starved animals the changes characteristic of the life cycle have been inhibited and they have remained at practically the same stage while other members of the same original generation have given rise to twelve generations of descendants. As a matter of fact the animals have not actually remained at exactly the same stage during this time but their life has consisted of alternating progressions and regressions of slight extent as periods of feeding and starvation have alternated.

IV. Conclusion.

This partial starvation experiment affords an interesting contrast to the experiment in asexual breeding. The latter demonstrates that the animals may undergo senescence and rejuvenescence for generation after generation of asexual reproduction without any indications as yet of progressive senescence of the stock. The former, on the other hand, demonstrates that senescence may be prevented by partial starvation for at least a length of time equal to twelve generations and judging from the present indications both experiments may be continued indefinitely, although the partial starvation experiment will finally be terminated by accidental losses, since there is of course no increase in the number of animals such as occurs in the breeding experiment.

Senescence in these animals is evidently associated with growth and rejuvenescence with reduction and reconstitution and neither has any necessary relation to sexual reproduction. In papers referred to above the writer has advanced the view that senescence in its simplest terms consists in a decrease in rate of metabolism resulting from the proportional decrease in amount of the metabolic substratum and from changes in the substratum which retard to the chemical reactions of metabolism. Rejuvenescence on the other hand is an increase in rate of metabolism resulting from the removal of inactive or less active substance and from changes in the substratum which permit a higher rate of reaction. Growth and differentiation bring about senescence and reduction and reconstitution bring about rejuvenescence. The facts of the present paper constitute further evidence in support of this view and show not only that the rejuvenescence of old animals is possible without reproduction of any kind but also that senescence can be prevented or at least retarded so as to be inappreciable for a long period of time by preventing growth and the later stages of differentiation.

SUMMARY.

1. *Planaria velata* has been bred asexually through thirteen generations in less than three years without any indications of progressive senescence in the stock. In each generation the animals have passed through the following cycle: reconstitution and rejuvenescence in the cysts, emergence as physiologically young, small animals, growth and senescence with feeding, cessation of feeding, fragmentation and encystment. During the period of breeding none of the animals have ever become sexually mature.

2. Starvation and reduction being about an increase in rate of metabolism and the reduced animals after renewed feeding are in the same physiological condition as young growing animals and are again capable of growth and senescence.

3. Senescence has been inhibited or so far retarded as to be inappreciable in a stock of *Planaria velata* during more than two years by partial starvation. During this time the animals have been kept at approximately the same size and in approximately the same physiological condition, viz., that of half grown animals, and no reproduction has occurred. During the same period another stock of animals collected at the same time and originally in the same generation has been fed and bred asexually and has passed through twelve generations.

4. In this species neither sexual nor asexual reproduction is necessary for the production of young individuals from old. Senescence is associated with growth and differentiation and rejuvenescence with reduction and reconstitution. In the asexual cycle senescence and rejuvenescence alternate and apparently balance each other, at least during thirteen generations and probably will continue to do so indefinitely.

HULL ZOÖLOGICAL LABORATORY,
UNIVERSITY OF CHICAGO.

AN EXPERIMENTAL STUDY OF THE BEHAVIOR AGREEMENT AMONG THE ANIMALS OF AN ANIMAL COMMUNITY.

VICTOR E. SHELFORD.

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I. INTRODUCTION.

On the basis of literature, naturalistic observation, and preliminary experiments the writer has several times stated ('13) that a physiological agreement exists among the animals of animal communities. The object of this investigation was to determine the extent and character of such agreement with particular reference to the rapids community of a large creek. It is the purpose of this paper to show that, considering the community as a whole, there is (1) a *general agreement* in reactions to certain factors, (2) *disagreement* in *respect* to factors differing in intensity *vertically* and (3) a *sharp difference* between *different* communities.

The rapids community was selected for detailed study because it was anticipated that the animals were governed mainly by mechanical stimuli which lend themselves to experiment more readily than many others. The pool community was studied in a *preliminary* way to bring out the difference between different communities.

Over two hundred experiments were performed by Chas. W. Finley and the writer working independently. It was originally hoped that these might be used in this discussion but the difficulty of adjusting conditions to which eight or more entirely different animal species can respond, is great, and finally the use of the first lot of experiments as a basis for comparison was decided to be impracticable. This was due to faulty conditions which gave bad results in the case of one or more species. However these experiments gave a close knowledge of the behavior of each species, so that when accurate methods were devised only sufficient experiments to give typical results proved necessary. These show experiments were conducted with much care and are the only ones presented.

II. MATERIAL.

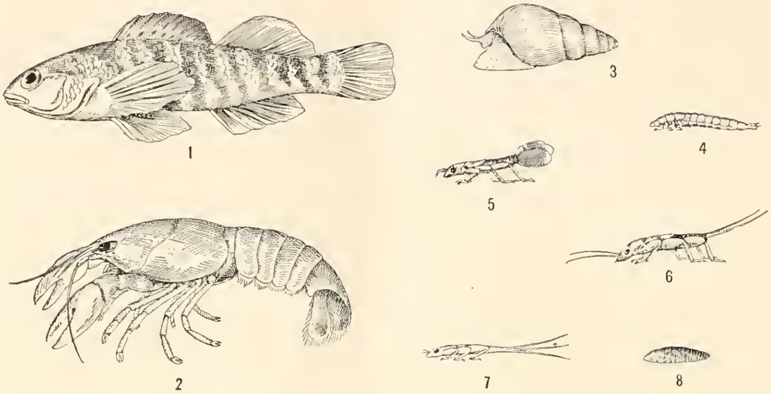
The material used were the species found habitually in rapids and pools of Hickory Creek at New Lenox, Ill. (Shelford '13). All the material used in the show experiments was collected in late September, October, and November, 1913. It was kept in as near natural conditions as possible in running lake Michigan water with gases at saturation. Most of the material used had been collected within three or four days but in a few cases material one week old was used. Several of the species in question occur outside of habitats of the type from which they were collected for these experiments. These relations are given below.

Rapids Community.

Etheostoma and *Cambarus* occur among and under stones. *Goniobasis* on stones, *Hydropsyche* on and under stones. The remainder under stones.

Species, Usual Habitat Rapids	Occasionally in	Rarely in
Etheostoma coeruelum Raf.	Pools of streams	Lakes and large streams
Cambarus virilis Hag.	Lakes	Lake Mich. (Harris '03)
Goniobasis livescens Me.	Sandy bot lakes	Veg. pool (Shelford '13)
Hydropsyche sp.	On shells in moderate current	Veg. in moderate current

Argia putrida		
Perla sp.		
Heptageninae		Shores large lakes.
Psephenus		Shores large lakes.



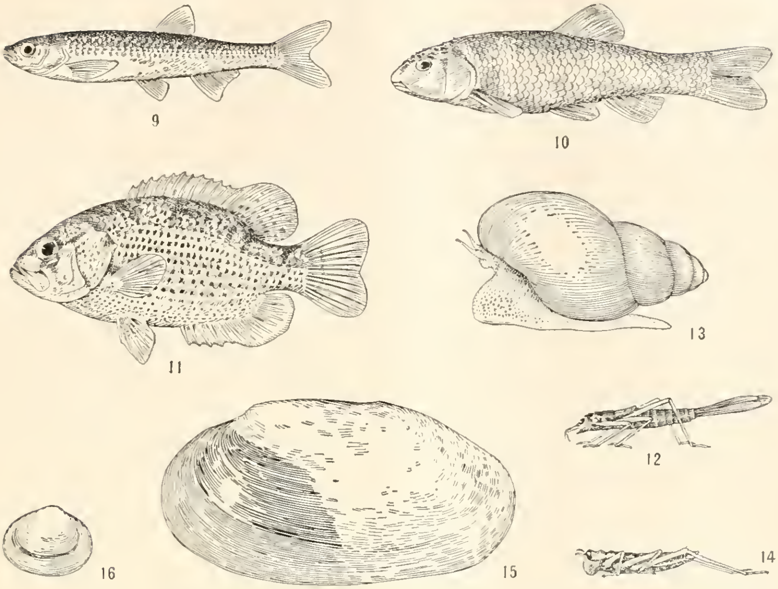
FIGS. 1–8. General form of rapids animals. Drawn on the same scale; all about natural size; seen from the side slightly above. 1, the rainbow darter (*Etheostoma coeruleum* Raf.); 2, crayfish (juvenile) (*Cambarus virilis* Hag.); 3, snail (*Goniobasis livescens* Mke.); 4, caddis worm (*Hydropsyche*); 5, damsel fly nymph (*Argia sp.*); 6, stone fly nymph (*Perla sp.*); 7, mayfly nymph (*Heptageninæ*); 8, water penny (*Psephenus sp.*).

POOL COMMUNITY.

The fishes live in the open water or in the shade of the scattered vegetation. Calopteryx rests on the vegetation. The rest burrow in the bottom. The species studied were not selected carefully as representative but were merely collected from pools.

Species, Usual Habitat Sand-bottomed Pools,	Occasionally on or in	Rarely in
Notropis atherinoides Raf.	Mud bottom	Lakes and ponds (Forbes and Richardson '08).
Hybopsis kentuckiensis Raf.	Mud bottom	Lakes and ponds.
Ambloplites rupestris Raf.	Large streams.	
Calopteryx sp.		
Campeloma subsolidum Ant.	Mud bottom	Among vegetation.
Anodontoides ferussacianus Lea.	Lakes	
Sphaerium striatinum (?)	Lake Mich. (11 meters).	

Side views of each of the animals (Figs. 1–16) studied show the radically different ways in which the animals receive stimuli such as current, horizontal light, etc.



Figs. 9–16. General form of the pool animals. 9, Shiner (*Notropis atherinoides* Raf.); 10, River chub (*Hybopsis kentuckiensis* Raf.); 11, Rock bass (*Ambloplites rupestris* Raf.) 12, Damsel fly nymph (*Calopteryx maculata* Beauv.); 13, River snail (*Campeloma subsolidum* Ant.) 14, Burrowing dragon fly nymph (*Macromia sp.*); 15, Mussel (*Anodontoides ferussacianus* Lea) 16, Small bivalve (*Sphaerium sp*). The fishes were juvenile. All are drawn on the same scale.

III. REACTIONS TO CURRENT.

1. Methods.

The tests were made in an Allee Straight Current apparatus, Fig. 17, the trough of which is 11 cm. wide, 7 cm. deep and 68 cm. long. Pieces of screen (32 meshes per inch) were placed across each end adjacent to the round reservoirs. The screens

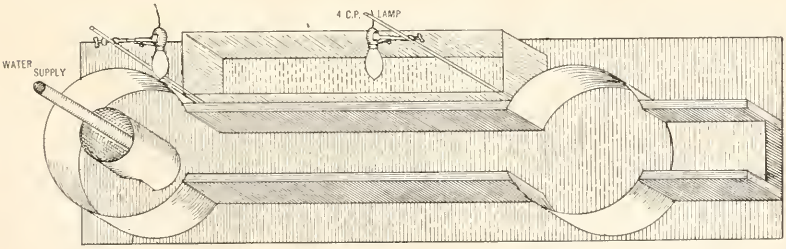


Fig. 17. Allee's straight current apparatus.

confined the animals in the central portion of the apparatus. Thirty-three centimeters above the bottom of the trough and 12–15 cm. from the respective ends were two 4-c.-p. carbon filament lamps. The control box was placed alongside the current trough and kept cool by streams of water.

Water flowed into the left-hand well from the supply pipe. The rates of flow were determined by measuring the amount of water that flowed through the trough in cubic centimeters per second. This was divided by the average area of the cross section of the water flowing through the trough, which gives the velocity in cm. per second (Finley's method). The velocity is determined by (*a*) the volume flowing into upper well per unit of time, (*b*) depth of water in trough, and (*c*) angle of slope.

VELOCITIES USED AND FACTORS CONTROLLING THEM.

Volume per sec.	Depth in Cm.	Degrees Deviation from Level	Velocity in Cm. per Second
200–250 c.c.	4.0–5.0	0	4–6
500–600 c.c.	4.5–5.0	1	10–12
500–600 c.c.	2.5–3.5	2.5	16–20

With very few exceptions five individuals were used. In each case the animals were poured into the center of the trough and readings begun after the animals had adjusted themselves to the current. This time differed greatly with different species, to a less extent with different lots of the same species. The differences between different species are due largely to different speeds of movement. Variations of the second kind were in strong current and due to the particular way in which the animals floated against the lower screen. The length of time before the first reading is given in general terms for each species.

2. SPECIFIC PECULIARITIES OF BEHAVIOR IN WATER CURRENT.

(Time before first reading given in brackets.)

Rapids Community.

Etheostoma (Fig. 1) [15–30 sec.]. Rest on bottom head up stream, move by darts, positively thigmotactic, often rest against lower screen and often forced against it in very strong current.

Cambarus (Fig. 2) [15–30 sec.]. Creep on bottom.

Goniobasis (Fig. 3) [30–40 min.]. Amount of activity is largely determined by current and light. Controls show little or no activity.

Hydropsyche (Fig. 4) [2–10 min.]. Strongly thigmotactic; the majority often did not leave the screen, and in some experiments they showed little positive orientation so several experiments were performed and an experiment which represented our general experience with them was selected for tabulation. Their tubes nearly always face the current in the rapids of streams (Wesenberg-Lund '11) and in experimental conditions where they are allowed to spin. They appear to have a greater efficiency in the current than any other of the species studied. When poured into a 60 cm. per sec. current, one out of three individuals succeeded in obtaining silk attachment and moved upstream 1 cm. per min. on a comparatively smooth wooden bottom. With the tubes once constructed they are secure against anything but floating objects.

Argia (Fig. 5) [2–5 min.]. Awkward in current. Orients well in very weak current due to large gill plates which act like a weather vane.

Perla (Fig. 6) and *Heptageninae* (Fig. 7) [5–30 sec.]. Both good runners; both crouch close to the bottom, especially the latter.

Psephenus (Fig. 8) [3–15 min.]. Slow to orient but very efficient in current.

Pool Community.

Notropis (Fig. 9) [immediately]. Swims constantly.

Hybopsis (Fig. 10) [immediately]. Swims and rests on bottom with head up stream.

Ambloplites (Fig. 11) [immediately]. Swims constantly; in strong current the fishes are thrown sidewise against the lower screen and cannot dislodge themselves.

Calopteryx (Fig. 12). Variable time to first reading—began when one left lower screen.

Campeloma (Fig. 13) [30 min.]. Good efficiency against current on account of large foot. Inactive in very strong current.

Macromia (Fig. 14). Apparently indefinite. Sometimes moving up or down in the trough; frequently resting for long periods ith the posterior end up stream.

Anodontoides (Fig. 15). Moves in the direction headed.
Sphærium (Fig. 16). Moves in the direction headed.

3. Method of Reading.

At the time of each reading the number of animals headed up stream within approximately 16 degrees of the direction of the current was counted positive. Those headed down stream were counted negative; those not falling within an arc of approximately 32 degrees of the total possible orientations for both positive and negative were counted as indefinite. Mollusks withdrawn *within their shells* and other animals *lodged against the screen* were counted as inactive or out of the experiment. Readings were taken fifteen seconds apart; fifteen seconds being sufficient time for individuals of all the species to orient at least once, most of them several times. In nearly all cases twenty readings were taken. With the exception of the snails, ten readings were taken, then the animals were disturbed, loosened from their footing, and after a short period they were read ten times again.

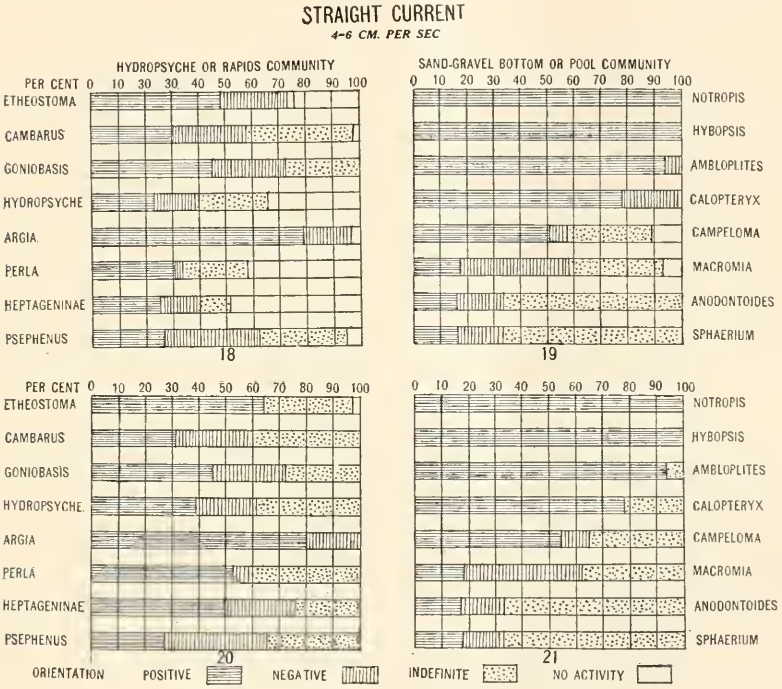
4. Progress Up Stream.

The preliminary experiments, especially those of Mr. Finley, showed that all the species make definite progress up stream. This results from (*a*) *positive orientation* and (*b*) movement. The amount of movement up stream differs for different species and for the same species under different conditions. Thus positive orientation, the first essential to up stream movement is the more significant.

5. Typical Results.

The diagrams illustrate typical *orientation* results, in per cent. of total. The upper diagrams (Fig. 18, 19, 22, 23, 26, 27) give per cent. positive, negative, indifferent and inactive (or on the screen, shown blank). Since inactive individuals and individuals on the screen cannot be regarded as responding to the current, the lower diagrams (Figs. 20, 21, 24, 25, 28, 29) give per cent. of *active* individuals showing positive, negative, and indefinite orientation. The data on the *active* individuals were used as a basis for comparison. Since *Anodontoides* and *Sphærium* move in the direction headed, the diagrams are of perfect chance indefiniteness, counting an arc of thirty two degrees of the possible circle of orientation as covering respectively positive and negative trials.

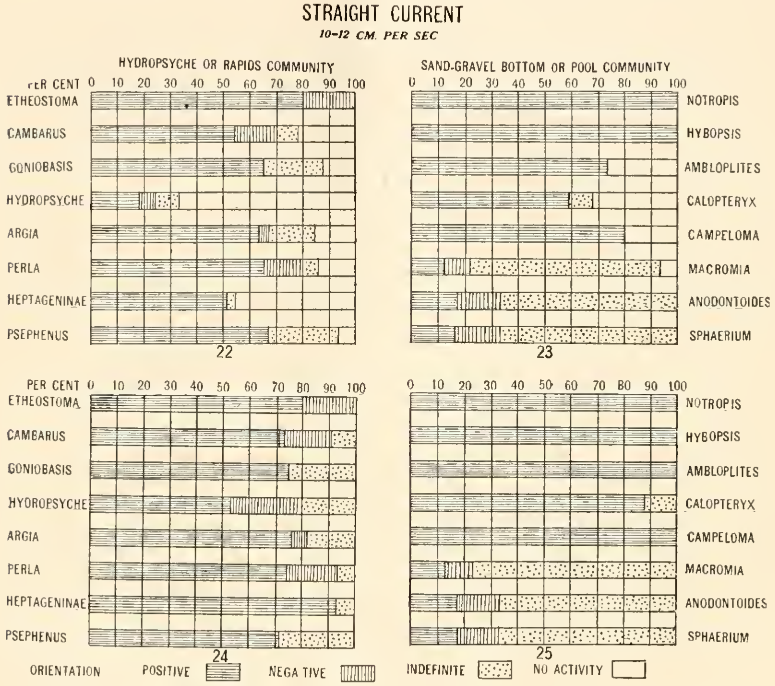
Comparing first the reactions of the *rapids* animals to the different velocities, we note that the positive orientations in the 4–6 cm. per sec. current are only a little greater than the negative and indifferent, excepting *Argia* which is thrown into line by the action of the current on the gill plates. The amount of activity was different for the different species.



FIGS. 18–21. Showing reactions to 4–6 cm. per sec, current in per cent. positive, negative, indifferent, and inactive (18–19) and in per cent. *active individuals* positive, negative, and indifferent (20–21). The rapids community (18) shows a large percentage of inactivity while the percentage of active positive (20) averages less than 50. The animals of the pool community were nearly all active. The first four non-burrowing species are strongly positive. It thus appears that 4–6 cm. per sec. is near the optimum for pool species. Temperature of water in experiments: Rapids animals 16° C.; pool animals 9° C.

In the 10–12 cm. per sec. current positive orientations (Figs. 22–24) are increased at the expense of the negative and indefinite while the same difference is further emphasized in the 16–20 cm. per sec. current and this without any material increase in the number of individuals resting on the screen.

16–20 cm. per sec. represents the flow to which the swift stream species appear best adapted. In this current the per cent. of active is more nearly the same for the different species (compare Fig. 28 and 24 with 20). These figures show a *remarkable uniformity of positive reaction*, over 93 per cent. for all but *Psephenus* and *Hydropsyche*, which are *most efficient* in clinging and less active otherwise. They are thus *ecologically equivalent* to the rest of the animals living under stones.



FIGS. 22–25. Showing reactions to 10–12 cm. current in per cent. positive, negative, indifferent, and inactive (22–23) and per cent. of active individuals positive, negative, and indefinite (24–25). Here there is *more* activity among the rapids animals and *less* among the pool animals than in the 4–6 current. The percentage of active positive is much higher in the case of the rapids animals. Temperature as in Figs. 18–21.

Comparing the *rapids* community with the *pool* community which was studied only briefly and the results added here to make the work on the rapids community clearer, we note that the greatest activity was apparently in the 4–6 cm. per sec. current (compare Figs. 19–23 and 27). None of the animals were washed against the screen. All were active except *Anodontoides* and *Sphærium*, which moved in course of 24 hours but in the direction in which they happened to be headed. The condition indicated in the diagram is for a chance orientation allowing 16 deg. on either side of the direction of the current as respectively positive and negative.

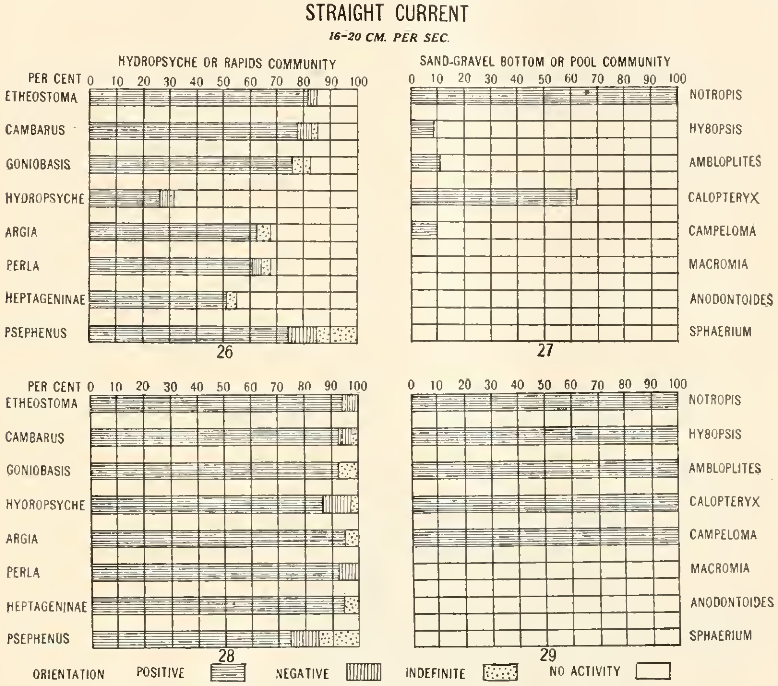


FIG. 26–29. Showing reactions to 16–20 cm. current in per cent. positive, negative, indefinite, and inactive (26–27) and per cent. of active individuals positive, negative, and indefinite (28–29). There was a large percentage of activity among the rapids animals and a small one among the pool animals, due in the case of the latter to inefficiency in the current. The rapids animals here show the greatest per cent. of active individuals positive (compare with Figs. 18–25). Temperature as for Figs. 18–20.

In the 10–12 cm. current (Fig. 23) the amount of activity still remains large but the wide rock bass was forced against the lower screen. *Campeloma* was less active. *Macromia* and *Calopteryx* were washed against the screen. *Anodontoides* and *Sphærium* showed activity as usual.

In the 16–20 cm. per sec. current only *Notropis* held out against the current for the five minutes during which the readings were taken. The other fish were swept against the screen very soon as were the other animals except *Anodontoides* which was inactive. Thus judging from (*a*) the amount of activity, (*b*) the efficiency and (*c*) the number of positive orientations, the 4–6 cm. per sec. current is probably nearest the optimum for the pool community.

IV. REACTIONS TO BOTTOM.

1. Method.

The tests were made in a dead black dark room under a hood of black sateen which permitted observation from between the symmetrically placed 4-c.-p. lamps clamped in a narrow slit (Fig. 30). The experimental boxes were two bread pans 10x31x50 cm. The bottom of one was entirely covered with beeswax, one half of the other with sand and the other half with wax into which the Warsaw Co.'s quartz dust numbers ½, 1,

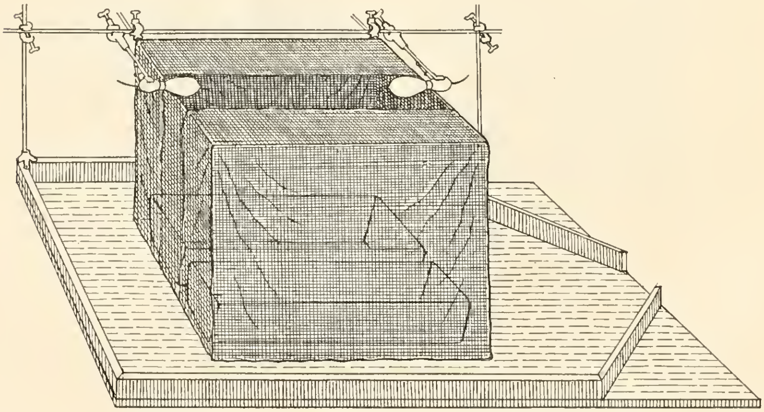


Fig. 30. Showing the water tray and hood in which the pans were placed in the study of reactions to bottom. The observations were made through the slit between the lights.

1½, 2, 2½ had been beaten to give a surface like stone. These two pans were placed side by side beneath the lamps which were 25 cm. apart and 28 cm. above the bottom. Water flowed around the pans and kept them at a temp. of 14° C. Water in the pans 1 to 2 cm. deep.

2. Specific Peculiarities.

Rapids Community.

Etheostoma and *Cambarus* readings every 30 sec. begun at once.

Goniobasis: 40 watt tungsten lamps were substituted to increase activity and the experiment was read every half hour (due to inactivity). The animals were placed in a row on the boundary between sand and hard material, in some cases so that they would extend and come in contact with the sand in others, with the hard material. When they became active, they crept from the sand to the hard bottom in all cases and usually turned back when moving from the hard bottom to sand. The reaction to sand and hard bottom in this species was influenced by reactions to gravity because while the sand and hard bottom in the experiment were at the same level the foot settled into the sand enough to make the hard bottom higher and the mollusks tend to crawl upward. The results are legitimate because in their natural habitat the animals can avoid sand by this means.

Hydropsyche. Readings every minute, begun after 3 min. They often turned back on reaching the sand and were more active while on it.

Argia. Readings every minute, begun after three minutes.

Perla. Readings every minute, begun after one minute.

Heptageninae. Readings every minute, begun after one min. Turn back on reaching the sand.

Psephenus. Readings every minute, begun after five minutes. The animals are evidently much irritated by the sand as they wave the thin margins of their bodies about, walk high on their legs and when once in contact with the hard bottom they come to rest and cling for days if not loosened.

Pool Community.

Notropis, *Hybopsis*, and *Ambloplites* apparently do not react to bottom when in small experimental boxes.

Calopteryx does not react sharply to bottom in these tests as it clings in vegetation particularly in sand bottomed pools but is frequently found on sand bottom.

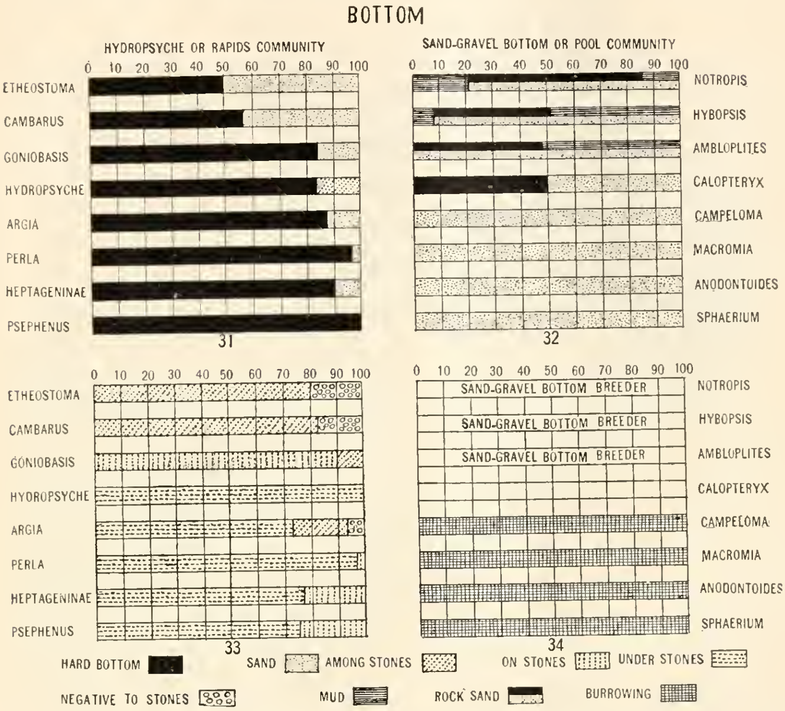
Campeloma, *Macromia*, *Anodontoides*, and *Sphærium*.—After creeping about for some time in the unnatural conditions of the experiment, come to rest, burrow in the sand, in most cases with a small portion of the body protruding. *Sphærium* burrows well only in the presence of a current.

3. Typical Results.

In the rapids community we note that there is a striking avoidance of sand by the animals living below the stones; less striking by those living on and among the stones. There is then a general agreement in the preference for hard surface and avoidance of sand. The animals of the pools all show a large preference for sand especially those living on the bottom; *Calopteryx* in the preliminary experiments showed no preference for either end but is for some reason commonly associated with sand. Since the fishes did not react to bottom in such small space it was necessary to draw, by way of predicting the character of response, data calculated from collections by Forbes and Richardson ('08). Here relations to mud and sand and rock are not clearly separated but the preference clearly includes sand for the majority of cases. The data in Fig. 34 shows the extent to which the tabulated animals burrow.

4. Reactions to Stones.

The reactions of the animals to stones were tested in the apparatus described for the bottom experiments. Two pans with waxed bottoms were placed side by side under the hood. In one half of one was placed a number of irregular pieces of quartz 1 × 2 inches and a number of pieces about ¼ of an inch in diameter. Ten individuals of each rapids species were placed in each pan and left in entire darkness. A one candle power lamp was turned on for the readings. The percentage of animals found under, on and among stones is shown in Fig. 33. The preference for stones was strong. Only the darters, crayfishes and *Argia* showed an avoidance of the stones in 20 per cent. or less of the trials. All the other species were among the stones, either on or under as indicated in the diagram.



FIGS. 31–34. Show the reactions to hard *vs.* sand bottom and to loose stones *vs.* wax bottom in per cent. of total. Fig. 31 shows the per cent. of rapids animals on sand and hard bottom, a large preference for the latter being evident. In the case of the pool fishes results of the experiments were unsatisfactory and as further tests had to be abandoned on account of cold weather the data of Forbes and Richardson ('08) is included to indicate what the probable results of experimentation will be. It will be noted that the preference is quite generally for sand, rock and mud occupying a much smaller portion than sand. Fig. 33 shows the relation to rocks on a wax bottom. Negative reaction to rocks is small. A striking agreement is shown in the general preference for stones. Fig. 34 shows the relation of the animals of the pool community to sand bottom with reference to burrowing. Here again the breeding data of the fish is taken from literature to indicate what might be found experimentally. Experiments performed at 14° C.

Fig. 34 shows further probable relations of the pool animals in such bottom experiments. The fishes usually bury their eggs and other species excepting *Calopteryx* bury the body in the sand.

V. REACTIONS TO LIGHT.

1. Specific Peculiarities—General Results of preliminary Experiments performed.

A large number of experiments was performed with light. Diffuse day light, tungsten lamps, Nernst lamps, with and without the cylindrical lens were used so as to obtain variations in direction and intensity. Since some of the animals react to intensity, some to direction and not to intensity, and since some readily *move* out of strong light while others tend to *stay out* of it, special methods were demanded.

The general characters of the reactions of different species are given below.

Etheostoma appears indefinite to light of the intensities used. Individuals make no recognizable response to either direction or intensity.

Cambarus does not react sharply to ordinary differences of intensity or to direction. In general they appear slightly negative to strong daylight often resting with the anterior end in the lighter parts. In an intensity gradient they back into the dark when the water or the apparatus are jarred which may account for their apparent negativeness, under experimental conditions in which the surrounding medium is disturbed. Finley found that they turn back from white paper used in making records.

Goniobasis appears positive to direction and to all intensities of room light when intensity accompanies direction and in Fig. 38 is believed to be less strongly positive than the animals usually are due to slowness of movement.

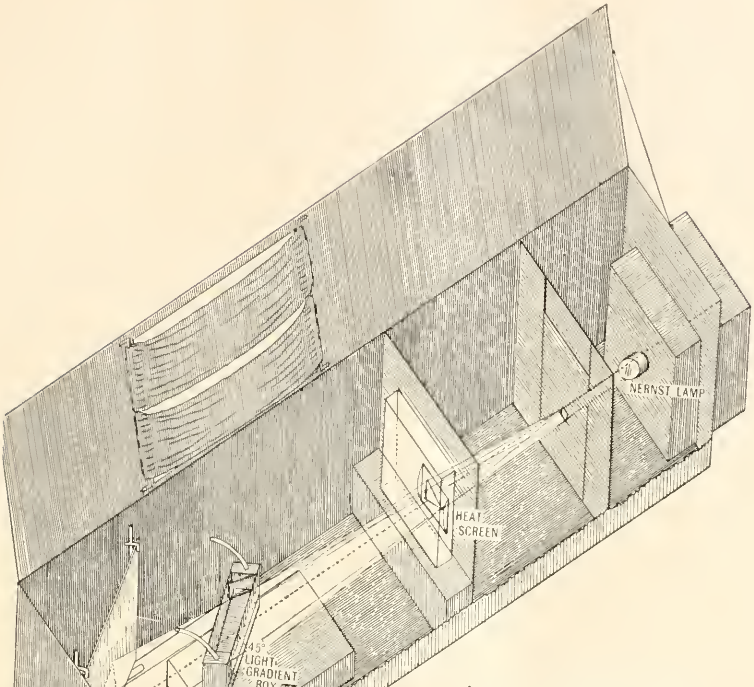
Hydropsyche is apparently indifferent to intensity; reacts positively to direction.

Argia does not react to intensity, orients negatively to direction reversing soon in some cases.

Perla starts into greater intensity and turns back; orients negatively to direction when a light is turned on.

Heptageninae—orient negatively to direction at first but quickly reverse in high intensity. Do not react clearly to intensity. Turn back on encountering strong light.

Psephenus is negative to direction, less so to intensity.



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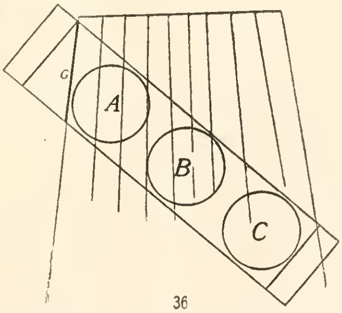


36

Fig. 35. Shows the light grader in which the experiments were performed. It is in all essentials like that described by Mast ('11), p. 61. A 132-watt lamp was used. The heat screen of distilled water, the gradient box and reflecting mirror are shown.

Fig. 36. Shows the plan of the experiments. The light gradient was focused at the horizontal line and the rays diverged slightly as indicated by the diverging lines. *G* is the glass plate to confine the animals from the dark corner. *A*, *B*, and *C* are the positions in which the bottomless cylinder was placed to confine the animals, before readings. The portion of the box to the right of the *C* was in essential darkness.

Pool Animals.

Notropis swam quickly about but spent most of its time in the medium and light thirds.

The other two species of fish and *Calopteryx* spent most of their time in the darker third while the other species were not active.

2. METHODS OF SHOW TESTS AND TYPICAL RESULTS.

The final tests or show experiments were made in a Yerkes light grader such as is described by Mast ('11 p. 61), (fig. 35). A 132-watt Nernst lamp was used 50 cm. from the lens (with a triangle of 70 mm. base and 82 mm. altitude) and the experimental box (5 cm. wide by 25 cm. long) was turned at an angle of 45 degrees to the direction of the light and with the nearest corner at the focal point of the lens and at the left end of the gradient field. A glass plate (*G* of Fig. 36) was placed across the corner to prevent the animals from moving into the darkness. The experimental box had glass sides and metal ends. All parts not made from glass were painted dead black and the light which

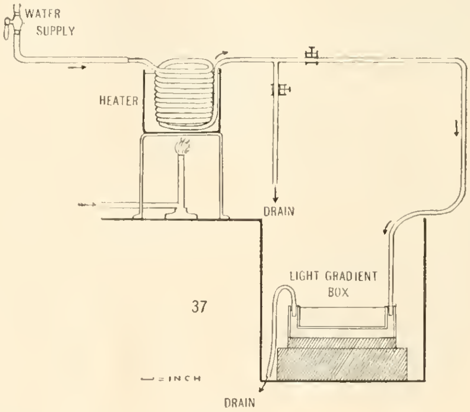
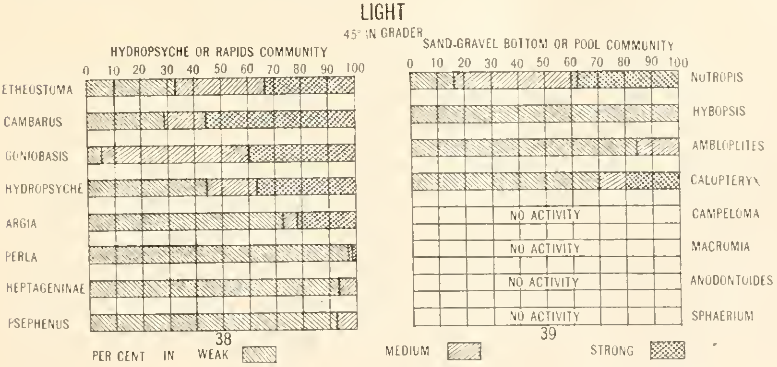


FIG. 37. Showing the relation of the hollow-walled glass-sided experimental box. The water came from the supply pipe, passed through the coil which rested in a vessel of water kept hot by a bunsen burner. The flow and gas were readily adjusted to give any desired temperature within experimental needs. The water passed from the coil to the hollow wall of the light box. It was necessary to empty the box often and when this was done the flow was temporarily turned out through the drain nearest the coil without changing the rate of flow.

passed through the box was reflected by a 45-degree mirror into the end of the grader box against dead black paper. The experimental box was made from copper with hollow bottom and ends which could be attached to a water tap by means of a hose. (Fig. 37). The water in these tests was kept at 14°C. by passing it through a coil immersed in water kept hot by a Bunsen burner (Fig. 37).

Aside from the general facts stated below and culled from the preliminary experiments all the experiments were performed with 5 individuals of each species placed in the 45 deg. box in the following manner. A small board was placed in front of the lens so as to shut off all light from the experimental box. A bottomless glass cylinder just fitting inside the box was placed in the position *A* of Fig. 36. Five specimens were poured into the cylinder. A board with a hood and 1-c.-p. lamp which lighted the sheet of paper on which the records were made but which threw very little light into the room was used in recording results and when all was in readiness the board in front of the lens was dropped and the cylinder removed in the same motion of the arm. Readings of the position of the animals in the thirds of the box were taken every 30 sec. up to a total of at least forty readings. In the case of some animals like *Psephenus* which move less rapidly after a short time, the number of readings was



FIGS. 38 AND 39. Showing the reactions of the animals in the light gradient in percentage in the thirds. Note differences related to the level at which the animals live. Experiments performed at 14° C.

increased and the time between them lengthened until the animals took up a characteristic position. The experiment was repeated with five other individuals confined in the center positions (*B* of Fig. 36) and again with five more confined in the dark portion (*C* of Fig. 36). The last 20 readings of each series *A*, *B*, *C*, were averaged to give the results shown in Figs. 38 and 39. In Fig. 38, we note that (1) the animals living under the stones in rapid water selected the darker portion of the gradient box; (2) *Hydropsyche* which is found under and on stones (in algæ) is less negative to the intensities used than those always under the stones; (3) *Goniobasis* which always lives on stones is more positive than any of the others; (4) *Cambarus* in these undisturbed conditions showed a slight excess percentage in the strongest light; (5) the darters were indifferent remaining in the third in which they were confined.

The animals of the pool community behaved very differently. *Notropis* was quite positive while the other fishes and *Calopteryx* which are associated with vegetation were quite negative. The Mollusca and *Macromia* were inactive.

VI. SUMMARY AND DISCUSSION.

Figs. 40 and 41 are introduced to show the character of the *agreement* and *disagreement* in the rapids community and the fact that the pool community is different and remains *unsolved*. Noting first Fig. 40, we see a noteworthy agreement in reaction to bottom (a preference for *hard bottom* which means *avoidance of sand*) and to current. Those living on or under stones (including *Hydropsyche* were found largely on stones in algæ) were under stones in general *darkness*. *Goniobasis* which lives on stones was found on stones in the experiments. *Etheostoma* and *Cambarus* which live among stones are found among stones. Thus we have vertical disagreement in the matter of relation to bottom. Turning to reactions to light we find a comparable difference. Animals living beneath stones show a preference for weak light, those on stones medium light, and those among stones strong light. If we were to study out the community in full we would find that reactions to many other factors would be of importance. The formation of associations (Wodsedalek, '12) no doubt is of importance. There is agreement in reaction to factors of prime importance and disagreement in respect to factors differing strikingly in the different situations in which the animals are living within the community.

The diagram for the pool species is introduced to show how strikingly it differs from that of the rapids community. Though agreement is not indicated here, our experience with the reactions of pool fishes and invertebrates to chemical differences in water, suggests that such differences may be of much importance to all the species.

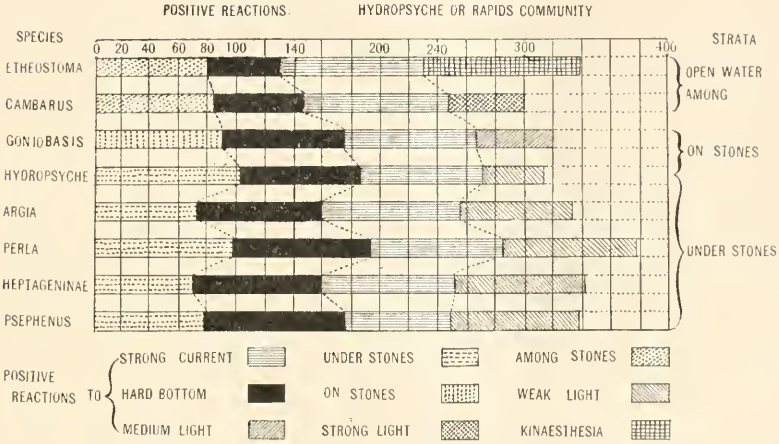


FIG. 40. Showing the agreement and disagreement of reaction of the rapids community. Note agreement of reaction to bottom and current and disagreement of two other reactions, related to the level at which the animals live. Each reaction is represented on a scale of 100 and if no other factors entered in the total should be 400 and the space all occupied. For strong agreement in positive reactions to stones see Fig. 33, p. 307.

The difference emphasized by the presence of two types of reactions not shown in the other charts, namely, a strong preference for bottom involving sand (see Fig. 32 for details in case of the fishes) and burrowing which are reactions not shown by the rapids species at all. The non-burrowing species are positive to current, the burrowing species do not respond within ordinary lengths of time. The reactions to light show much more sharp negativeness than in the case of darters and crayfish. The community is clearly unsolved as far as agreement is concerned and a large amount of experimentation would be necessary to determine suitable tests for these animals and then all the animals from both communities should be put through all the tests new and old. A series of new tests must be added for each new aquatic community and all the old tests must be so modified as to secure good response from all the animals. Thus the labor involved in comparing a number of communities is great.

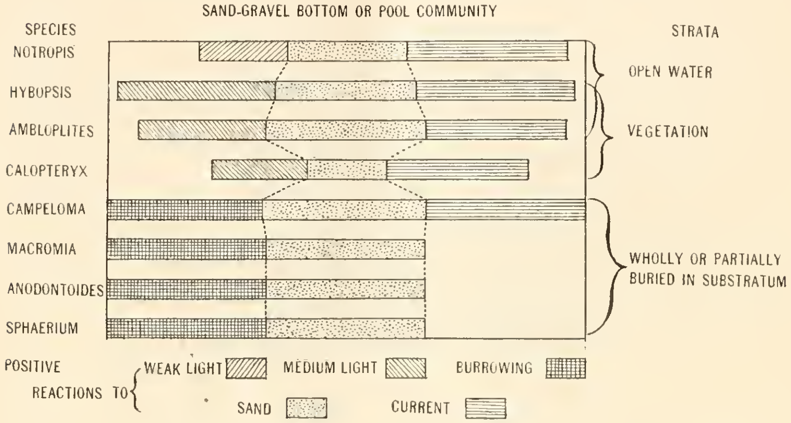

SAND-GRAVEL BOTTOM OR POOL COMMUNITY
SPECIES
STRATA
NOTROPIS
HYBOPSIS
AMBLOPLITES
CALOPTERYX
CAMPELOMA
MACROMIA
ANODONTOIDES
SPHAERIUM
OPEN WATER
VEGETATION
WHOLLY OR PARTIALLY BURIED IN SUBSTRATUM
POSITIVE REACTIONS TO
WEAK LIGHT
MEDIUM LIGHT
BURROWING
SAND
CURRENT

FIG. 41. Showing suggestions as to the probable agreement and disagreement of the reaction of the animals of the unsolved pool community on a basis of a total of 300, introduced to show the striking differences between communities.

VII. SUMMARY OF CONCLUSION.

1. The animals of an animal community are in agreement in the reaction to certain intensities of two or more factors. These reactions may be used to designate them. Thus the rapids community may be designated as *litho-rheotactic* meaning that the animals are arranged with reference to current and stones of considerable size.

2. Animals living in the *same* or comparable situations within the community habitat are in agreement with respect to factors not concerned in the general agreement and the animals of *different* situations react differently to these additional factors. Similar differences are the physiological basis for strata and consocies though the small number of species makes the latter not easily distinguishable here.

3. Single species found in any community occur in other situations where they are governed chiefly by stimuli toward which there is *not* agreement of reaction throughout the communty to which they primarily belong.

Hull Zoological Laboratory,
University of Chicago,
February 20, 1914.

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Vol. XXVI. *June, 1914.* *No. 6*

BIOLOGICAL BULLETIN

THE MARINE BIOLOGICAL LABORATORY

Sixteenth Report

FOR THE YEAR 1913

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I. TRUSTEES

EX OFFICIO

F. R. Lillie, *Director*, The University of Chicago.
Gilman A. Drew, *Assistant Director*, Marine Biological Laboratory.
D. Blakely Hoar, *Treasurer*, 161 Devonshire Street, Boston, Mass.
Gary N. Calkins, *Clerk of the Corporation*, Columbia University.

TO SERVE UNTIL 1917

S. F. Clarke..........Williams College.
Charles A. Coolidge...Ames Building, Boston, Mass.
C. R. Crane..........2559 Michigan Boulevard, Chicago, Ill.,
President of the Board.

Alfred G. Mayer	Carnegie Institution.
C. E. McClung	University of Pennsylvania.
T. H. Morgan	Columbia University.
Erwin F. Smith	United States Department of Agriculture.
E. B. Wilson	Columbia University.

TO SERVE UNTIL 1916

H. H. Donaldson	Wistar Institute of Anatomy and Biology.
M. J. Greenman	Wistar Institute of Anatomy and Biology.
C. W. Hargitt	Syracuse University.
H. S. Jennings	Johns Hopkins University.
George Lefevre	University of Missouri, Secretary of the Board.
A. P. Mathews	University of Chicago.
G. H. Parker	Harvard University.

TO SERVE UNTIL 1915

H. C. Bumpus	University of Wisconsin.
R. A. Harper	Columbia University.
W. A. Locy	Northwestern University.
Jacques Loeb	The Rockefeller Institute for Medical Research.
F. P. Mall	Johns Hopkins University.
George T. Moore	Missouri Botanical Garden, St. Louis.
L. L. Nunn	Telluride, Colo.
John C. Phillips	299 Berkeley Street, Boston, Mass.

TO SERVE UNTIL 1914

Cornelia M. Clapp	Mount Holyoke College.
E. G. Conklin	Princeton University.
Ross G. Harrison	Yale University.
Camillus G. Kidder	27 William Street, New York City.
M. M. Metcalf	Oberlin College.
William Patten	Dartmouth College.
Jacob Reighard	University of Michigan.
W. B. Scott	Princeton University.

II. ACT OF INCORPORATION

No. 3170.

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our LORD ONE THOUSAND, EIGHT HUNDRED and EIGHTY-EIGHT.

HENRY B. PIERCE,
Secretary of the Commonwealth.

[SEAL.]

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

. The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory, in Woods Hole, Mass., at 12 o'clock noon, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk, who shall be, *ex officio*, members of the Board of Trustees, and Trustees as hereinafter provided. At the annual meeting to be held in 1897, not more than twenty-four Trustees shall be chosen, who shall be divided into four classes, to serve one, two, three, and four years, respectively, and thereafter not more than eight Trustees shall be chosen annually for the term of four years. These officers shall hold their respective offices until others are chosen and qualified in their stead. The Director and Assistant Director, who shall be chosen by the Trustees, shall also be Trustees, *ex officio*.

II. Special meetings of the members may be called by the Trustees, to be held in Boston or in Woods Hole at such time and place as may be designated.

III. The Clerk shall give notice of meetings of the members by publication in some daily newspaper published in Boston at least fifteen days before such meeting, and in case of a special meeting the notice shall state the purpose for which it is called.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. The Trustees shall have the control and management of the affairs of the Corporation; they shall present a report of its condition at every annual meeting; they shall elect one of their number President and may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

VI. Meetings of the Trustees shall be called by the President, or by any two Trustees, and the Secretary shall give notice thereof by written or printed notice sent to each Trustee by mail, postpaid. Seven Trustees shall constitute a quorum for the transaction of business. The Board of Trustees shall have power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient.

VII. The President shall annually appoint two Trustees, who shall constitute a committee on finance, to examine from time to time the books and accounts of the Treasurer, and to audit his accounts at the close of the year. No investments of the funds of the Corporation shall be made by the Treasurer except approved by the finance committee in writing.

VIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be given to the Boston Society of Natural History, or some similar public institution, on such terms as may then be agreed upon.

IX. These By-Laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-Laws will be acted upon.

X. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

IV. TREASURER'S REPORT

Cash Receipts and Disbursements
For the Year Ending December 31, 1913

Receipts

Cash on hand January 1, 1913	$ 4,788.70	
Annual dues	1,012.00	
Biological Bulletin	2,031.46	
Boats	5.00	
Carpenter shop	12.89	
Chemical department	3.17	
Charles R. Crane	21,000.00	
Donations	35.00	
Dormitory, stone building	338.25	
Dormitory, Whitman cottage	236.50	
Fish trap	337.48	
Instruction, botany	350.00	
Instruction, embryology	1,150.00	
Instruction, physiology	450.00	
Instruction, zoölogy	1,600.00	
Library	1.43	
Maintenance buildings and grounds	7.83	
Mess	12,152.26	
Mess extension	339.86	
Miscellaneous	373.24	
Pile driver	35.00	
Research	2,575.00	
Supply department	14,554.40	$63,389.47

Payments

Administration	$ 6,932.18
Bath house	8.00
Biological Bulletin	2,344.13
Botany building	516.65
Boats	4,566.01
Carpenter shop	215.14
Chemical department	2,022.34

Dormitory, stone building	283.55	
Dormitory, Whitman cottage	547.10	
Fish trap	897.60	
Instruction, botany	1,125.00	
Instruction, embryology	454.31	
Instruction, physiology	650.00	
Instruction, zoölogy	1,081.00	
Lectures	48.35	
Library	1,177.41	
Maintenance buildings and grounds	4,477.27	
Mess	11,042.28	
Mess extension	3,971.12	
Miscellaneous	2,281.48	
New laboratory	740.44	
Oil house	436.86	
Philosophical lectures	100.00	
Pile driver	87.77	
Pumping station	1,890.39	
Store house	106.18	
Supply department	9,439.16	
Supply department, improvements	1,284.70	
Cash on hand January 1, 1914	4,663.05	$63,389.47

Cash Receipts and Disbursements on Account of Funds August 1, 1913, to January 1, 1914

Reserve Fund

Cash on hand August 1, 1913	$ 222.12	
Div. 6 shs. Am. Smelting & Refining Co. Pfd.	21.00	
Div. 8 shs. Gen. Elec. Co.	16.00	
Div. 14 shs. United Shoe Mach. Corp. Pfd.	5.25	
Interest on deposit	3.51	$ 267.88

Crocker Fund

Cash on hand August 1, 1913	88.79	
Div. 1 sh. Am. Tel. & Tel. Co.	2.00	
Div. 18 sh. Vermont & Mass. R. R. Co.	54.00	

Div. 1 sh. West Wnd St. Ry. Co.	1.75	
Div. 2½ sh. Gen. Elec. Co.	5.00	
	$ 151.54	
Payment of 2 scholarships	100.00	51.54

Library Fun

Cash on hand August 1, 1913	$ 191.04	
Div. 3 shs. Am. Tel. & Tel. Co.	6.00	
Div. 1 sh. Am. Smelting & Refining Co. Pfd.	3.50	
Div. 2½ shs. Gen. Elec. Co.	5.00	
Div. 5 shs. United Shoe Mach. Corp. Pfd.	1.87	207.41
Cash on January 1, 1914		$526.83

MARINE BIOLOGICAL LABORATORY INVESTMENTS (BOOK VALUE)

JANUARY 1, 1914

Reserve Fund

$3,000 Am. Tel. & Tel. Co. 4%	$2,921.25	
6 shs. Am. Smelting & Refining Co. Pfd.	732.00	
8 shs. Gen. Elec. Co.	972.05	
14 shs. United Shoe Mach. Corp. Pfd.	393.75	
Cash	267.88	
	$5,286.93	
Part of the above stocks and bonds is held as collateral for loan of	3,000.00	$2,286.93

Library Fund

3 shs. Am. Tel. & Tel. Co.	$ 381.18	
4/5 of $1,000 Am. Tel. & Tel. Co. 4%	779.00	
1 sh. Am. Smelting & Refining Co. Pfd.	122.00	
2½ shs. Gen. Elec. Co.	288.10	
5 shs. United Shoe Mach. Corp. Pfd.	140.63	
Cash	207.41	1,918.32

Lucretia Crocker Fund

18 shs. Vermont & Mass. R. R. Co.	$2,416.50	
1 sh. West End St. Ry. Co.	83.00	
1 sh. Am. Tel. & Tel. Co.	127.06	

1/5 of $1,000 Am. Tel. & Tel. Co. 4%	194.75	
2½ shs. Gen. Elec. Co.	323.85	
Cash	51.54	3,196.70
		$7,401.95

V. LIBRARIAN'S REPORT

AUGUST, 1913

With expenditures about the same, approximately $1,000, the library has been considerably improved in several ways since last year.

1. Most important is the gift from the Carnegie Institution of a complete set of publications relating to our work. There are very few laboratories in this list, so the placing of the library on the mailing list is a decided gain. In this way about 85 valuable monographs, not otherwise available, have been received.

2. Last year, about this time, a strong representation was placed before a number of publishing firms, of the advantages to them of sending here copies of works on science and education. The response was very prompt and generous; and as a result about 150 books have been added representing a value of over $250. A word to publishers on the part of authors will now be sufficient to explain to them the value of such advertisement as is furnished by a place on our shelves.

3. In response to letters enclosing lists showing missing parts and asking aid in completing our sets, we have been able to fill in some bad gaps; and are still receiving assistance here. The lists are available to anyone who will help.

4. An important departure, which we hope will establish a precedent, has been the gift of subscriptions to new journals by members of the corporation: *The Popular Science Monthly*, *The American Journal of Anatomy*, *The American Naturalist*, *The Annual Bulletin of the Zoölogical Museum of the Imp. Acad. of Science, St. Petersburg*, and *The Journal of The Society for Experimental Biology and Medicine.* Drs. A. Mayer, Bumpus, and Knower have each given $10 a year for five years toward journals, either for new subscriptions or missing parts of back sets. Much

extension of this assistance is desirable as more journals are greatly needed and we are only spending at the present time about $700 for this purpose.

5. We have added by exchange: *Bulletin Scientifique de La France et Belgique*, Vols. 26 to 46; *Mittheilungen aus dem Naturhistorisches Museum in Hamburg*, Vols. 7 to 29; also a complete series of the *Monist* and the *Wilson Bulletin* from Oberlin, O.

6. The reprint collection has been greatly improved by rearrangement and considerable addition. We must, however, appeal again for gifts to this collection. It is in much demand and we are quite dependent on authors to remember this.

Finally, the librarian feels obliged to urge the great importance of now engaging a trained assistant with ability to carry on, throughout the year, a systematic campaign to develop the library. This work should be done under the general direction of the librarian, by maintaining a business-like correspondence in regard to accessions, exchanges, missing parts, etc. It is the only way in which we can steadily and adequately improve. The peculiarly free methods used in this library demand constant study of the shelves, catalogues, etc., to take account of losses, needed repairs and disarrangements caused by the free access to the shelves. This is especially necessary after the summer session; otherwise the entire collection becomes more or less disorganized and chaotic and there is inevitable loss from year to year. The binding, catalogues, special sets, repairs, reprints have to be constantly watched and kept strictly up to date, to make the material which we have more available. We have been suffering from very inadequate and irregular assistance.

Dr. Drew's work on the catalogues and binding and the enterprise of his office in pushing some of the schemes which have improved things have demonstrated what can and should be done.

This summer, as soon as Dr. Drew's office force was diverted into its regular work, we were again obliged to drop back to carrying through the routine demands of the busy season, with what aid we could secure. Much more could and should be done through a permanent assistant librarian keeping behind the library work winter and summer.

I should recommend that such an appointment be made in advance of the move to our permenent quarters; since this should be supervised by an experienced person, and because there is important preliminary work now awaiting attention.

H. Mc. E. Knower,
Librarian.

VI. THE DIRECTOR'S REPORT

To the Trustees of the Marine Biological Laboratory:

Gentlemen: I have the honor to transmit herewith a report of the twenty-sixth session of the Marine Biological Laboratory. The year just drawing to a close has been in many respects the most successful and encouraging since the foundation of the Laboratory. Not only has there been a great increase in attendance which has tasked our accommodations beyond our previous estimates of the uttermost, but the spirit of research has never been more intense or on a higher plane, and many interesting results have been obtained. If these could in some way be exhibited together I feel confident that they would amount to a really great increase in the resources of biological facts and principles. This matter is, however, intangible and impossible to estimate at close range. Even after publication the results of a particular piece of investigation are not capable of immediate appraisal; and but little of last season's work is yet published. Moreover in our absolutely free method of organization with no prescription as to material, or subject matter, or method of investigation, and no restriction as to time or place of publication, one can gain only the most general impression of the results actually secured by so large a body of investigators. I can only feel, and express the opinion, that the laboratory is maintaining its best traditions in these respects.

Turning to the record of the principal events of the year: the attendance of the investigators was in excess of the largest previous attendance by nearly one third; the actual figures are 122 in 1913, 93 in 1912, 82 in 1911, 62 in 1910. The number of students in courses was 69, making a total attendance of investigators and students of 191, as compared with 160 in 1912, 147 in 1911 and 126 in 1910. The number of subscribing institutions was 30 in 1913, as compared with 29 in 1912, 25 in 1911, 24 in 1910, 20 in 1909, 18 in 1908 and 16 in 1907. The list is given on p. 339. Of these Barnard College, the Crocker Research Fund of Columbia University, Harvard University, Kansas State Agricultural College and Radcliffe College are new subscriptions. The receipts from the subscribing institutions and students' fees were $6,160 in 1913, as compared with $5,175 in 1912, $4,574.99 in 1911, $4,150 in 1910 and $3,700.35 in 1909. The receipts from the supply department were $14,554.40 in 1913, as compared with $13,966.35 in 1912, $10,303.61 in 1911, $9,300.58 in 1910, and $8,549.55 in 1909.

At the beginning of 1913 a new system of book-keeping was established with the aid of Dr. Drew. While this may cause some difficulty in comparison of the treasurer's report for the current year with the preceding reports, it gives a much more accurate idea of the real classification of expenses and the cost of departments, and it is hoped that it may furnish the basis for more complete comparisons in the future. The book-keeping is now done at Wood's Hole, under the supervision of the treasurer, instead of in Boston as formerly; this is a great aid in administration, as it enables the executive officers to keep closer account of receipts and expenditures.

Great improvements were made in the mess in preparation for the past summer session. The dining-room was enlarged by about 50 per cent., a wide covered verandah was built around three sides of it; the kitchen was much enlarged and its ventilation improved and a new laundry built. The expense of these improvements, $5,720.16, was provided for partly by the earnings of the mess $1,581.70, partly by subscriptions amounting to $2,147.33 from a considerable number of subscribers, and partly by current funds of the Laboratory to which the mess now stands indebted in the sum of $1,991.13.

The large increase of investigators was accommodated by utilizing the Kidder Annex, the Yacht Club building, and every available corner of the old buildings. The progress of the new building was therefore particularly inspiriting in its promise of relief from overcrowding. We purpose to publish an illustrated account of this building in the BIOLOGICAL BULLETIN at an early date. We shall therefore deal briefly with it here:

At the winter meeting of the board of trustees in 1909 a committee was appointed to prepare plans for a permanent building of fireproof construction, to accommodate the library and to include a number of research laboratories equipped for more delicate researches and capable of being used at all seasons of the year. The president of the board, Mr. Charles R. Crane, promised to contribute the necessary funds for construction, and our fellow member of the board, Mr. Charles A. Coolidge, the distinguished architect, agreed to furnish the plans of the building as his contribution. Such concurrence of generous support has rarely happened in the history of any institution. Plans were carefully studied by the committee in charge, and an interior arrangement best suited to our needs was agreed on; Mr. Coolidge then prepared various exterior plans. After much study and discussion it was finally agreed that the simplest plan of architecture was most in keeping with our traditions and the location. In the summer of 1912 the plans were complete. They were submitted to many of the workers at the laboratory for criticism and suggestion and were finally approved by the board. The contracts were signed that winter and construction was begun in February, 1913.

The location decided on was selected as part of a plan for the location of several more buildings of permanent construction. The new building faces south on the Woods Hole Harbor. It is constructed of tapestry brick with stone trimmings; in form rectangular 92 by 50 feet, three stories and a high basement. The height of the three stories, the proportions of the openings, the construction of the cornice, and the nature of the materials combine to produce a pleasing effect of great dignity. The building is now nearly completed and will be ready for occupancy in March, 1914.

Hitherto the work of the Laboratory has been carried on in buildings of an avowedly temporary character. The completion of the first permanent building, therefore, marks a stage in the growth of the laboratory deserving of especial recognition. I, therefore, recommend to the board, at the suggestion of the president, that special exercises of dedication be held to commemorate the event early in the next summer session, and that a committee be appointed to make arrangements therefor. The significance of the event relates not only to the development of our institution, but also especially to the dedication of so adequate an equipment for the purposes of research in pure science outside of our universities.

Among the clearly defined tendencies in the growth of our institution is the increase in numbers of our membership. As I pointed out in my last report the living accommodations in Woods Hole for transient workers at the Laboratory are actually inadequate. We need more dormitory space, especially for women. The suggestion in my last report that one of the smaller laboratory buildings might be made over as a dormitory for women seems now to be impracticable, if the increase in attendance of last summer is any indication of what to expect in the future.

There is a great need of cottages for the families of workers at the Laboratory. Those available are held at exorbitant rents. As the personnel of the investigators is really our prime consideration this is a problem of importance. By coöperation it should be possible for such persons to build bungalows without financial loss. But unless some plan is organized from the Laboratory nothing is likely to be done, because the people interested are not in touch with one another. It would be an important aid to the cause of science if someone would erect a group of small cottages that might be rented for a small return on the investment at from $100 to $200 for the season.

We have provided a fine library room well equipped for several times the present number of volumes. The development of the library should now be made a special problem. The appointment of a library assistant for the entire year would be a most desirable step in this direction. Until this is done, the development of the library is likely to be spasmodic and slower than need be. I refer to the report of the librarian (p. 324) on this subject.

A new and larger lecture hall is another immediate need and the improvement of the rooming house for employees of the mess yet another.

The equipment of the new laboratory has been provided for by an additional gift from Mr. Crane, and the executive officers have proceeded with the necessary plans in order to avoid delay in the opening of the building. The most important part of the preparation, viz: provision for adequate pumping facilities, is completely arranged for. It involves construction of a wharf on the harbor front to carry a supply pipe out to deep water, but also useful for other laboratory purposes, and the installation of pumps and the power plant. After considerable inquiry we decided on hard rubber pumps of such construction that no metal can come in contact with the sea-water. We believe that all sources of metal contamination of the sea-water in the system of pumping and distribution have been avoided. The valves in the system are of special lead, manufactured by the Crane Valve Company, thus avoiding a source of metal contamination in the use of brass or bronze valves. The power will be furnished by electric motors with a reserve gasoline engine. Other details of equipment need not be included in this report.

At the last meeting of the board of trustees the director and assistant director were authorized to proceed with plans for the improvement of the water front. Surveys were accordingly made, and plans prepared for the erection of sea-walls both on the harbor and eel-pond frontage; wharves in connection with these improvement were also planned, and filling and grading in order to utilize the space in the best possible way. Such part of these plans as required state permits were presented to the harbor and land commission of the state of Massachusetts and the permits secured. The drawings of these projects are herewith presented.

It is not necessary to proceed with all of this work at once; but it is essential that anything undertaken should be part of a general plan, and this is the idea we have had in mind in planning so much at once.

We still have before us the necessity of an endowment before we can feel certain that the operation of the Laboratory can be continued uninterruptedly. The estimates for the year 1914 show a probable deficit of about $20,000, a very small sum considering the magnitude and significance of our operations. An endowment of $500,000 would permanently insure our present status at least, and I feel very strongly that every effort should be made to raise such a sum before we prepare for farther expansion.

I. THE STAFF

1913

F. R. LILLIE, DIRECTOR,
Professor of Embryology, The University of Chicago.

GILMAN A. DREW, ASSISTANT DIRECTOR,
Marine Biological Laboratory.

ZOÖLOGY

I. INVESTIGATION

Zoölogy and Embryology

GARY N. CALKINS	Professor of Protozoölogy, Columbia University.
E. G. CONKLIN	Professor of Zoölogy, Princeton University.
GILMAN A. DREW	Assistant Director, Marine Biological Laboratory.
GEORGE LEFEVRE	Professor of Zoölogy, University of Missouri.
FRANK R. LILLIE	Professor of Embryology, The University of Chicago.
T. H. MORGAN	Professor of Experimental Zoölogy, Columbia University.
E. B. WILSON	Professor of Zoölogy, Columbia University.

II. INSTRUCTION

CASWELL GRAVE	Associate Professor of Zoölogy, Johns Hopkins University.
GEORGE A. BAITSELL	Dean and Professor of Biology, Central College.
RAYMOND BINFORD	Professor of Biology, Guilford College.
J. K. BREITENBECKER	Fellow in Zoölogy, University of Chicago.
E. J. LUND	Bruce Fellow in Zoölogy, Johns Hopkins University.
T. S. PAINTER	Graduate Student of Zoölogy, Yale University.

EMBRYOLOGY

I. INVESTIGATION (See Zoology)

II. INSTRUCTION

Gilman A. Drew	Assistant Director, Marine Biological Laboratory.
Lorande L. Woodruff	Assistant Professor of Biology, Yale University.
A. L. Treadwell	Professor of Biology, Vassar College.
Robert A. Budington	Associate Professor of Zoölogy, Oberlin College.

PHYSIOLOGY

I. INVESTIGATION

Albert P. Mathews	Professor of Physiological Chemistry, The University of Chicago.
R. S. Lillie	Assistant Professor of Experimental Biology, University of Pennsylvania.
Harold C. Bradley	Assistant Professor of Physiological Chemistry, University of Wisconsin.

II. INSTRUCTION

Ralph S. Lillie	Assistant Professor of Experimental Biology, University of Pennsylvania.
Walter E. Garrey	Associate Professor of Physiology, Washington University Medical School.
Frank P. Knowlton	Professor of Physiology, Syracuse University.
Edward B. Meigs	Associate in Physiology, Wistar Institute of Anatomy and Biology.

PHILOSOPHICAL ASPECTS OF BIOLOGY AND ALLIED SCIENCES

LECTURES

Edward G. Spaulding	Assistant Professor of Philosophy, Princeton University.

BOTANY

George T. Moore	Engelmann Professor of Botany, Washington University.
George R. Lyman	Assistant Professor of Botany, Dartmouth College.
B. M. Duggar	Professor of Plant Physiology, Washington University.

Ivey F. Lewis	Assistant Professor of Botany, University of Wisconsin.
W. J. Robbins	Assistant in Plant Physiology, Cornell University.
R. H. Colley	Instructor in Botany, Dartmouth College.
A. R. Davis	Lockland Research Fellow, Shaw School of Botany.

LIBRARY

H. McE. Knower	University of Cincinnati, Librarian.

CHEMICAL SUPPLIES

Oliver S. Strong	College of Physicians and Surgeons, New York City, Chemist.

G. M. Gray	Curator of Supply Department.
A. L. Leathers	Collector.
John Veeder	Cockswain.
F. M. MacNaught	Business Assistant.

2. INVESTIGATORS AND STUDENTS

1913

INVESTIGATORS 1913

ZOÖLOGY

Independent Investigators

Allee, W. C., Instructor in Zoölogy, Williams College.
Baitsell, George A., Graduate Student, Yale University.
Beckwith, Cora J., Instructor in Biology, Vassar College.
Binford, Raymond, Professor of Biology, Guilford College.
Boring, Alice M., Associate Professor of Zoölogy, University of Maine.
Breitenbecker, J. K., Instructor in Biology, Western Reserve University.
Browne, Ethel N., Instructor in Biology, Dana Hall, Wellesley College.
Budington, Robert A., Associate Professor of Zoölogy, Oberlin College.
Bullock, F. D., Associate in Cancer Research, Columbia University.
Calkins, Gary N., Professor of Protozoölogy, Columbia University.
Chambers, Robert, Assistant Professor of Histology and Comparative Anatomy, University of Cincinnati.
Child, C. M., Associate Professor of Zoölogy, University of Chicago.

Clapp, Cornelia M., Professor of Zoölogy, Mount Holyoke College.
Conklin, E. G., Professor of Biology, Princeton University.
Crampton, H. E., Professor of Zoölogy, Barnard College, Columbia University.
Drew, Gilman A., Assistant Director, Marine Biological Laboratory.
Edwards, Dayton J., Tutor in Physiology, College of the City of New York.
Glaser, O. C., Junior Professor of Zoölogy, University of Michigan.
Goldfarb, A. J., Instructor in Zoölogy, College of the City of New York.
Grave, Caswell, Professor of Zoölogy, Johns Hopkins University.
Grave, B. H., Professor of Biology, Knox College, Galesburg, Ill.
Gregory, Louise H., Instructor in Zoölogy, Barnard College.
Harvey, E. N., Instructor in Physiology, Princeton University.
Hegner, R. W., Assistant Professor of Zoölogy, University of Michigan.
Hogue, Mary J., Instructor in Zoölogy, Mount Holyoke College.
Hyde, R. R., Assistant Professor of Physiology and Zoölogy, Indiana State Normal School.
Jackson, Robert T., Professor of Paleontology, Harvard University.
Just, E. E., Professor of Zoölogy, Howard University.
Knower, H. McE., Professor of Anatomy, University of Cincinnati.
Lefevre, George, Professor of Zoölogy, University of Missouri.
Lillie, Frank R., Professor of Embryology, University of Chicago.
Lund, E. J., Adam T. Bruce Fellow, Johns Hopkins University.
McClung, C. E., Professor of Zoölogy, University of Pennsylvania.
McGregor, J. H., Professor of Zoölogy, Columbia University.
Mall, F. P., Professor of Anatomy, Johns Hopkins University.
Malone, E. F., Assistant Professor of Anatomy, University of Cincinnati.
Morgan, T. H., Professor of Experimental Zoölogy, Columbia University.
Morrill, C. V., Instructor in Anatomy, New York University.
Morse, Edward S., Director, Peabody Museum, Salem, Mass.
Newman, H. H., Associate Professor of Zoölogy, University of Chicago.
Painter, T. S., Instructor in Zoölogy, Roanoke College.
Pappenheimer, A. M., Associate in Pathology, Columbia University.
Parmenter, C. S., Vice-president and Professor of Zoölogy, Baker University, Baldwin, Kansas.
Paton, Stewart, Lecturer in Biology, Princeton University.
Patterson, J. T., Professor of Zoölogy, University of Texas.
Reinke, E. E., Fellow in Zoölogy, Princeton University.
Robertson, W. R. B., Assistant Professor of Zoölogy, University of Kansas.
Shorey, Marian L., Professor of Biology, Milwaukee-Downer College.
Shull, A. Franklin, Assistant Professor of Zoölogy, University of Michigan.
Spaeth, R. A., Research Student, Harvard University.
Spaulding, E. G., Assistant Professor of Philosophy, Princeton University.
Stockard, C. R., Professor of Anatomy, Cornell Medical College
Strong, O. S., Instructor in Anatomy, Columbia University.
Strong, R. M., Instructor in Zoölogy, University of Chicago.
Thompson, Caroline B., Associate Professor of Zoölogy, Wellesley College.
Treadwell, A. L., Professor of Biology, Vassar College.
Van Cleave, H. N., Instructor in Zoölogy, University of Illinois.
Wilson, E. B., Professor of Zoölogy, Columbia University.
Woodruff, L. L., Assistant Professor of Biology, Yale University.

Beginning Investigators

Bridges, Calvin B., Graduate Student, Columbia University.
Carver, Gail L., Professor of Biology, Mercer University.
Dexter, John S., Fellow in Zoölogy, Columbia University.
Faust, E. C., Research Assistant, University of Illinois.
Fish, J. Burton, Graduate Student, Columbia University.
Glaser, R. W., Bussey Institution, Forest Hills, Boston, Mass.
Goodrich, H. B., Assistant in Zoölogy, Columbia University.
Hayden, Margaret A., Instructor in Biology, Carnegie Institute of Technology.
Heilbrunn, L. V., Laboratory Assistant in Zoölogy, University of Chicago.
Hoge, Mildred A., Graduate Student, Columbia University.
Isaacs, Raphael, Assistant in Zoölogy and Embryology, University of Cincinnati.
Linkins, R. H., Assistant in Zoölogy, University of Illinois.
Lynch, Clara J., Instructor in Zoölogy, Smith College.
MacDowell, E. C., Graduate Student, Harvard University.
Morris, Margaret, 53 Edgehill Road, New Haven, Conn.
Packard, Charles, Assistant in Zoölogy, Columbia University.
Shumway, Waldo, University Scholar in Zoölogy, Columbia University.
Stark, Mary B., Graduate Student, Columbia University.
Sturtevant, A. H., Graduate Student, Columbia University.
Wardwell, E. H., Assistant in Biology, Princeton University.
Wheeler, Isabel, 18 The Hattersley, Toledo, Ohio.

PHYSIOLOGY

Independent Investigators

Bancroft, F. W., Associate Member in Department of Experimental Biology, Rockefeller Institute for Medical Research.
Bradley, H. C., Assistant Professor of Physiological Chemistry, University of Wisconsin.
Donaldson, H. H., Wistar Institute of Anatomy and Biology.
Ewald, W. F., Fellow, Rockefeller Institute for Medical Research.
Garrey, W. E., Associate Professor of Physiology, Washington University.
Hyde, Ida H., Professor of Physiology, University of Kansas.
Kite, G. L., Assistant in Physiological Chemistry, University of Chicago.
Knowlton, F. P., Professor of Physiology, Syracuse University.
Lillie, R. S., Assistant Professor of Experimental Zoölogy, University of Pennsylvania.
Loeb, Jacques, Head of Department of Experimental Biology, Rockefeller Institute for Medical Research.
Mathews, A. P., Professor of Physiological Chemistry, University of Chicago.
Meigs, E. B., Wistar Institute of Anatomy and Biology.
Moore, A. H., Associate Professor of Physiology, Bryn Mawr College.
Morse, Max W., Trinity College, Hartford, Conn.
Tashiro, Shiro, Associate in Physiology, University of Chicago.
Wasteneys, Hardolph, Associate in Experimental Biology, Rockefeller Institute for Medical Research.
Wherry, W. B., Associate Professor of Bacteriology, University of Cincinna

Beginning Investigators

Adams, H. S., Fellow in Chemistry, University of Chicago.
Cattell, McKeen, Student, Columbia University.
Gould, H. N., Fellow in Biology, Princeton University.
Kanda, Sakyo, Fellow in Psychology, Clark University.
Lloyd, Dorothy J., 16 Ampton Road, Edghaston, Birmingham, England.
Oliver, Wade W., Graduate Student, University of Cincinnati.
Stringer, Caroline E., Head of Biology Department, Omaha High School.

BOTANY

Independent

Duggar, B. M., Research Professor of Plant Physiology, Washington University.
Garber, John F., Head of Botany Department, Yeatman High School, St. Louis, Mo.
Hibbard, Rufus P., Instructor in Plant Physiology, Michigan Agricultural College.
Lewis, I. F., Assistant Professor of Botany, University of Wisconsin.
Lyman, George R., Assistant Professor of Botany, Dartmouth College.
Moore, George T., Director, Missouri Botanical Gardens.
Nichols, Susan P., Associate Professor of Botany, Oberlin College.
Osterhout, W. J. V., Professor of Botany, Harvard University.
Snow, Laetitia M., Associate Professor of Botany, Wellesley College.
Stomps, Theodor J., Professor of Cytology, University of Amsterdam.
Wuist, Elizabeth D., 2351 East 5th Street, Dayton, Ohio.

Beginning Investigators

Colley, R. H., Instructor in Biology, Dartmouth College.
Curtis, Otis F., Instructor in Botany, Cornell University.
Davis, A. R., Lackland Research Fellow, Washington University.
Foster, Goodwin L., Graduate Student, Dartmouth College.
Hopping, Aleita, Tottenville, Staten Island, New York.
Robbins, W. J., Instructor in Plant Physiology, Cornell University.
Roberts, Edith A., Instructor in Botany, Mount Holyoke College.

STUDENTS

1913

ZOÖLOGY

Abbott, Charles H., Student, Brown University.
Allen, Charles E., Student, Wabash College.
Atwood, Warren G., Student, Dartmouth College.
Baker, Florence I., Student, Carleton College.
Baumann, Emil J., Student, Yale University.
Beyer, Henry G., U. S. Navy, Retired.
Bobb, Thomas N., Instructor in Zoölogy, Northland College.
Bradley, Barbara, Technical Assistant in Zoölogy, University of Wisconsin.

Cashmann, Marguerite, Student, Syracuse University.
Clapp, Isabel C., Student, Oberlin College.
Daley, Mary W., Teacher of Physics, Dana Hall, Wellesley College.
Ewald, Marina, 78 Kaiserin Augusta Str., Berlin N. 10, Germany.
Harp, Margery B., Student, Syracuse University.
Heaps, Pearl I., Head of Biology Department, Western High School, Baltimore. Md.
Hoar, Carl S., Austin Teaching Fellow, Harvard University.
Keith, Gerald, Student, Amherst College.
Lewis, Elsie M., Student, Oberlin College.
Linton, Edwin S., Student, Washington and Jefferson College.
Mahnken, Florence V., 598 E. 167th Street, New York City.
McFarland, Helen J., Student, Bryn Mawr College.
Mallard, Agnes K., Teacher, Boston Elementary School.
Nelligan, Katherine, Student, Mount Holyoke College.
Patten, Mary W., Student, Goucher College.
Pinkerton, Mary B., 408 Warren Crescent, Norfolk, Va.
Plough, Harold H., Laboratory Asslstant, Amherst College.
Rieke, Benjamin W., Student, Carleton College.
Roberts, Amabel S., Madison, N. J.
Schulte, Anne M., Student, Sweet Briar College.
Stone, Phebe, Student, Goucher College.
Ware, Clara C., Hingham, Mass.
Wilson, Charlotta W., Technical Assistant, Johns Hopkins University.
Woglom, William H., Assistant Professor, Assigned to Cancer Research, Columbia University.

EMBRYOLOGY

Allen, Ezra, Professor of Biology, School of Pedagogy, Philadelphia.
Allen, George D., Instructor in Zoölogy, University of Minnesota.
Beers, Catherine V., Student, Northwestern University.
Behre, Elinor H., Instructor in Zoölogy, Sophie Newcomb College, Tulane University.
Burr, Harold S., Currier Fellow, Sheffield Scientific School, Yale University.
Cook, Dellar L., 28 Andrews Street, Woonsocket, R. I.
Farnam, Louise W., 43 Hillhouse Avenue, New Haven, Conn.
Field, Hazel E., Head of Science Department, Belhaven College, Jackson, Miss.
Gardner, Katherine, Student, Mount Holyoke College.
Gunther, Maude C., Instructor in Biology, Eastern High School, Washington, D. C.
Hance, Robert T., Assistant in Biology, University of Cincinnati.
Knight, Marian V., Demonstrator in Zoölogy, Smith College.
Lee, Barbara M., Teacher of Physics and Chemistry, High School, Puyallup, Wash.
Lowther, Florence deL., Graduate Student, Barnard College.
McMorland, Edward E., Assistant in Zoölogy, University of Missouri.
Martin, Bertha E., Graduate Student, University of Chicago.
May, H. G., Graduate Student, University of Rochester.
Meierhof, Harold L., Student, Columbia University.
Phipps, Charles F., Instructor, School of Education, University of Chicago.

Ruddiman, Marguerite, 441 Senator Street, Brooklyn, New York.
Turner, Clarence L., Ohio Wesleyan University, Delaware, Ohio.
Yocum, Harry B., Instructor, Wabash College.
Young, Donnell B., Laboratory Instructor, Amherst College.

PHYSIOLOGY

Angle, Brownie, 714 Washington Blvd., Kansas City, Kansas.
Duff, Dorothy, 49 Crescent Street, Montreal, Canada.
Kortright, W. H., Student, Syracuse University.
Mateer, John G., Student, Wooster University.
Olmsted, J. M. D., Assistant Professor of Biology, Richmond College.
Phillips, Ruth L., Instructor in Biology, Western College.
Root, Francis M., Graduate Student, Johns Hopkins University.
Trueblood, Ira C., Head of Biological Department, High School, Greencastle, Indiana.

BOTANY

Fink, Henri, St. Mathew's, Jefferson County, Kentucky.
Johnston, Sarah, 18 Franklin Street, Northampton, Mass.
Thompson, Bertha E., Instructor, Michigan Agricultural College.
Thurlow, Madge D., Goucher College.
Smith, Edith L., Assistant, West Roxbury High School.
Stewart, Mary W., Assistant, Barnard College, Columbia University.

3. TABULAR VIEW OF ATTENDANCE

	1910	1911	1912	1913
Investigators—Total	62	82	93	122
Independent				
Zoölogy	33	42	44	58
Physiology	11	18	14	17
Botany	9	8	10	11
Under Instruction				
Zoölogy	6	12	21	21
Physiology	2	2	2	7
Botany			2	7
Students—Total	64	65	67	69
Zoölogy	31	26	24	33
Embryology	10	20	15	22
Physiology	5	6	11	8
Botany	17	13	17	7
Total Attendance	126	147	160	191

INSTITUTIONS REPRESENTED				
Total			57	80
By investigators	26	37	43	50
By students	24	31	36	41
SCHOOLS AND ACADEMIES REPRESENTED				
By investigators	5	3	2	3
By students	6	9	1	6

4. SUBSCRIBING INSTITUTIONS, 1913

BARNARD COLLEGE.
BRYN MAWR COLLEGE.
CARLETON COLLEGE.
COLUMBIA UNIVERSITY.
CROCKER RESEARCH FUND OF COLUMBIA UNIVERSITY.
DARTMOUTH COLLEGE.
ELSE SERINGHAUS SCHOLARSHIP OF NORMAL COLLEGE.
GOUCHER COLLEGE.
HARVARD UNIVERSITY.
KANSAS STATE AGRICULTURAL COLLEGE.
LUCRETIA CROCKER SCHOLARSHIPS.
MOUNT HOLYOKE COLLEGE.
NORTHWESTERN UNIVERSITY.
OBERLIN COLLEGE.
PRINCETON UNIVERSITY.
RADCLIFFE COLLEGE.
ROCHESTER UNIVERSITY.
ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH.
SMITH COLLEGE.
SYRACUSE UNIVERSITY.
UNIVERSITY OF CHICAGO.
UNIVERSITY OF CINCINNATI.
UNIVERSITY OF ILLINOIS.
UNIVERSITY OF MICHIGAN.
UNIVERSITY OF MISSOURI.
UNIVERSITY OF PENNSYLVANIA.
VASSAR COLLEGE.
WELLESLEY COLLEGE.
WISTAR INSTITUTE OF ANATOMY AND BIOLOGY.
YALE UNIVERSITY.

5. EVENING LECTURES, 1913

H. E. Crampton	"Four Journeys among the Islands of Polynesia"	July 3.
A. G. Mayer	"Effects of the Electrolytes of Sea-water upon the Rate of Nerve Conduction in *Cassiopea*"	July 8.
John Auer	"Anaphylaxis"	July 11.
T. H. Morgan	"Heredity in *Drosophila*"	July 15.
Stewart Paton	"The National Campaign for Mental Hygiene in Relation to the Study of Human Activities"	July 18.
W. J. V. Osterhout	"Some Quantitative Researches on the Permeability of Plant Cells"	July 22.
E. S. Morse	"Mars and its Mysteries"	July 25.
W. E. Garrey	"Some Aspects of the Physiology of Cardiac Nerves"	July 29.
C. H. Eigenmann	"The Panama Problem and a Trip Through Colombia, South America"	August 1.
C. E. McClung	"Chromosome Individuality"	August 5.
C. R. Stockard	"Injurious Treatments of the Male, and the Influence on the Offspring"	August 8.
Charles T. Brues and M. J. Rosenau	"The Relation of the Stable Fly (*Stomaxys calcitrans*) to the Transmission of Infantile Paralysis"	August 12.

6. MEMBERS OF THE CORPORATION

1. Life Members, 1912.

Allis, Mr. E. P., Jr., Palais Carnoles, Menton, France.
Andrews, Mrs. Gwendolen Foulke, Baltimore, Md.
Billings, Mr. R. C., 66 Franklin St., Boston, Mass.
Carey, Mr. Arthur Astor, Fayerweather St., Boston, Mass.
Clarke, Prof. S. F., Williams College, Williamstown, Mass.
Conklin, Prof. Edwin G., Princeton University, Princeton, N. J.

Crane, Mr. C. R., 2559 Michigan Blvd., Chicago, Ill.
Davis, Major Henry M., Syracuse, N. Y.
Evans, Mrs. Glendower, 12 Otis Place, Boston, Mass.
Farlow, Prof. W. G., Harvard University, Cambridge, Mass.
Fay, Miss S. B., 88 Mt. Vernon St., Boston, Mass.
Folsom, Miss Amy, 88 Marlboro St., Boston, Mass.
Foot, Miss Katherine, 80 Madison Ave., New York City, N. Y.
Gardiner, Mrs. E. G., Woods Hole, Mass.
Gardiner, Miss Eugenia, 15 W. Cedar St., Boston, Mass.
Hannaman, Mr. Charles E., 103 1st St. Troy, N. Y.
Harrison, Ex-Provost C. C., University of Pennsylvania, Philadelphia, Pa.
Jackson, Miss M. C., 88 Marlboro St., Boston, Mass.
Jackson, Mr. Chas. C., 24 Congress St., Boston, Mass.
Kennedy, Mr. Geo. G., 284 Warren St., Roxbury, Mass.
Kidder, Mr. C. G., 27 William St., New York City, N. Y.
Kidder, Mr. Nathaniel T., Milton, Mass.
King, Mr. Chas. A.,
Lee, Mrs. Frederic S., 279 Madison Ave., New York City, N. Y.
Lowell, Mr. A. Lawrence, 171 Marlboro St., Boston, Mass.
Marrs, Mrs. Laura Norcross, 9 Commonwealth Ave., Boston, Mass.
Mason, E. F., 1 Walnut St., Boston, Mass.
Mason, Miss Ida M., 1 Walnut St., Boston, Mass.
Means, Mr. James Howard, 196 Beacon St., Boston, Mass.
Merriman, Mrs. Daniel, Worcester, Mass.
Minns, Miss Susan, 14 Louisburg Square, Boston, Mass.
Minns, Mr. Thomas, 14 Louisburg Square, Boston, Mass.
Minot, Dr. Chas. S., Harvard Medical School, Boston, Mass.
Mixter, Miss M. C., 241 Marlboro St., Boston, Mass.
Morgan, Mr. J. Pierpont, Jr., Wall and Broad Sts., New York City, N. Y.
Morgan, Prof. T. H., Columbia University, New York City, N. Y.
Morgan, Mrs. T. H., New York City, N. Y.
Noyes, Miss Eva J., 28 South Willow St., Montclair, N. J.
Nunn, Mr. Lucian L., Telluride, Colo.
Osborn, Prof. Henry F., American Museum of Natural History, New York.

Phillips, Dr. John C., 299 Berkeley St., Boston, Mass.
Phillips, Mrs. John C., 299 Berkeley St., Boston, Mass.
Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pa.
Pulsifer, Mr. W. H., Newton Center, Mass.
Rogers, Miss A. P., 5 Joy St., Boston, Mass.
Sears, Dr. Henry F., 420 Beacon St., Boston, Mass.
Shedd, Mr. E. A.,
Smith, Mrs. C. C., 286 Marlboro St., Boston, Mass.
Strobell, Miss E. C., 80 Madison Ave., New York City, N. Y.
Thorndike, Dr. Edward L., Teacher's College, Columbia University, New York City, N. Y.
Trelease, Prof. William, University of Illinois, Champaign, Ill.
Ware, Miss Mary L., 41 Brimmer St., Boston, Mass.
Warren, Mrs. S. D., 67 Mt. Vernon St., Boston, Mass.
Whitney, Mr. Henry M., Brookline, Mass.
Willcox, Miss Mary A., Wellesley College, Wellesley, Mass.
Wilmarth, Mrs. H. D., Elliott St., Jamaica Plain, Mass.
Williams, Mrs. Anna P., 505 Beacon St., Boston, Mass.
Wilson, Dr. E. B., Columbia University, New York City, N. Y.
Wilson, Prof. W. P., Philadelphia Museum, Philadelphia, Pa.

2. Members, August, 1913

Abbott, Prof. J. F., Washington University, St. Louis, Mo.
Abbott, Miss Margaret B., 413 W. Front Street, Plainfield, N. J.
Adams, Dr. C. F., University of Arkansas, Fayetteville, Ark.
Addison, Dr. W. H. F., Univ. of Pennsylvania, Med. Sch., Philadelphia.
Adkins, Mr. W. S., Department of Zoölogy, Columbia University, New York City.
Allen, Prof. Ezra, School of Pedagogy, Philadelphia, Pa.
Allyn, Dr. Harriet M.,
Alsburg, Dr. C. S., U. S. Department of Agriculture, Washington, D. C.

Baitsell, Dr. George A., Sheffield Scientific School, Yale University, New Haven, Conn.

Baker, Dr. E. H., 5727 Kimbark Avenue, Chicago, Illinois.
Bancroft, Dr. F. W., Rockefeller Institute, New York City.
Bardeen, Prof. C. R., Univ. of Wisconsin, Madison, Wis.
Beckwith, Miss Cora J., Vassar College, Poughkeepsie, N. Y.
Behre, Miss Elinor H., Sophie Newcomb College, Tulane Univ., New Orleans.
Beyer, Dr. H. C., Stoneleigh Court, Washington, D. C.
Bigelow, Prof. M. A., Teachers' College, Columbia University, N. Y. C.
Bigelow, Prof. R. P., Mass. Inst. Technology, Boston, Mass.
Binford, Dr. Raymond, Guilford College, S. C.
Box, Miss Cora May, Univ. of Cincinnati, Cincinnati, O.
Bradley, Dr. Harold S., Univ. of Wisconsin, Madison, Wis.
Browne, Miss Ethel N., Dana Hall, Wellesley College, Wellesley, Mass.
Buckingham, Miss Edith N., 342 Marlboro Street, Boston, Mass.
Budington, Prof. R. A., Oberlin College, Oberlin, Ohio.
Bumpus, Dr. H. C., University of Wisconsin, Madison, Wis.
Byrnes, Dr. Esther F., 193 Jefferson Avenue, Brooklyn, N. Y.

Calkins, Prof. Gary N., Columbia Univ., New York City.
Calvert, Prof. Philip P., Univ. of Pennsylvania, Philadelphia.
Carlson, Prof. A. J., Univ. of Chicago, Chicago, Ill.
Carver, Mr. Gail L., Mercer Univ., Macon, Ga.
Cary, Dr. L. R., Princeton Univ., Princeton, N. J.
Cattell, Prof. J. McKeen, Garrison-on-Hudson, New York.
Chambers, Dr. Robert, Univ. of Cincinnati, Cincinnati, Ohio.
Cheeseman, Dr. T. M., Garrison, N. Y.
Chester, Prof. Webster, Colby College, Waterville, Me.
Chidester, Dr. F. E., Rutgers College, New Brunswick, N. J.
Child, Prof. C. M., Univ. of Chicago, Chicago, Ill.
Clapp, Prof. Cornelia M., Mt. Holyoke College, South Hadley, Mass.
Clark, Dr. E. R., Johns Hopkins Univ., Baltimore, Md.
Coe, Prof. W. R., Yale University, New Haven, Conn.
Colton, Prof. H. S., Ardmore, Pa.
Cook, Miss Dellar L., 28 Andrews St., Woonsocket, R. I.
Comstock, Prof. J. H., Cornell University, Ithaca, N. Y.

Coolidge, Mr. C. A., Ames Building, Boston, Mass.
Crampton, Prof. H. E., Barnard College, Columbia Univ., New York City.
Crane, Mrs. C. R., 1559 Michigan Boulevard, Chicago, Ill.
Curtis, Prof. W. C., University of Missouri, Columbia, Mo.

Derick, Prof. Carrie M., McGill Univ., Montreal, Canada.
Dexter, Mr. H. S., Northland College, Ashland, Wis.
Dodds, Prof. G. S., Univ. of Missouri, Columbia, Mo.
Donaldson, Dr. H. H., Wistar Institute of Anat. and Biol., Philadelphia.
Dorrance, Miss Ann, Dorranceton, Pa.
Dorrance, Miss Frances, Dorranceton, Pa.
Drew, Prof. Gilman A., Marine Biological Laboratory, Woods Hole, Mass.
Duggar, Prof. B. M., Cornell Univ., Ithaca, New York.
Dungay, Dr. Neil S., Carleton College, Northfield, Minn.

Eaton, Prof. E. H., Hobart College, Geneva, New York.
Edwards, Mr. D. J., College of the City of New York, New York City.
Eigenmann, Prof. C. H., Univ. of Indiana, Bloomington, Ind.
Ewald, Dr. W. F., Rockefeller Institute, New York City.

Farnam, Miss Louise W., 43 Hillhouse Avenue, New Haven, Conn.
Ferguson, Prof. J. S., Cornell Univ. Medical School, New York City.
Field, Miss Hazel E., Belhaven College, Jackson, Miss.
Field, Prof. Irving, 5 Enfield Street, Worcester, Mass.
Fish, Mr. J. Burton, 883 Freeman Street, New York City.
Flanigen, Miss Ruth, Woodbury, New Jersey.
Furst, Mr. Clyde, Carnegie Foundation, 576 Fifth Ave., New York City.

Gage, Prof. S. H., Cornell Univ., Ithaca, New York.
Garrey, Prof. W. E., Washington Univ., St. Louis, Mo.
Gies, Prof. W. J., Columbia Univ., Dept. Physiological Chemistry, New York.
Glaser, Prof. O. C., Univ. of Michigan, Ann Arbor, Mich.

GLASER, MR. R. W., Bussey Institution, Forest Hills, Boston, Mass.

GOLDFARB, PROF. A. J., College of the City of New York, New York City.

GOODRICH, MR. H. B., Columbia Univ., Dept. Zoology, New York City.

GRAVE, DR. CASWELL, Johns Hopkins Univ., Baltimore, Md.

GREGORY, DR. LOUISE H., Barnard College, Columbia Univ., New York City.

GREENMAN, Dr. M. J., Wistar Institute of Anat. and Biol., Philadelphia.

GUNTHER, MISS MAUDE C., Eastern High School, Washington, D. C.

HARPER, PROF. R. A., Columbia Univ., New York City.

HAHN, Dr. C. W., High School of Commerce, New York City.

HALL, PROF. ROBERT W., Lehigh University, South Bethlehem, Pa.

HANCE, MR. ROBERT T., Univ. of Cincinnati, Cincinnati, Ohio.

HARGITT, DR. C. W., Syracuse Univ., Syracuse, New York.

HARRISON, MR. A. C., 660 Drexel Building, 5th & Chestnut Sts., Philadelphia.

HARMAN, DR. MARY T., Kansas State Agricultural College, Manhattan, Kans.

HARRISON, PROF. ROSS G., Yale University, New Haven, Conn.

HARVEY, PROF. B. C. H., Univ. of Chicago, Chicago, Ill.

HARVEY, DR. E. N., Princeton Univ., Princeton, N. J.

HAYDEN, MISS MARGARET A., Carnegie Institute of Technology, Pittsburgh.

HAYES, PROF. S. P., Mount Holyoke College, South Hadley, Mass.

HEATH, PROF. HAROLD, Stanford University, Calif.

HEGNER, PROF. R. W., Univ. of Michigan, Ann Arbor, Mich.

HOAR, MR. D. BLAKELY, 161 Devonshire Street, Boston, Mass.

HOGE, MISS MILDRED A., 509 W. 121st Street, New York City.

HOGUE, DR. MARY J., Mt. Holyoke College, South Hadley, Mass.

HOLMES, PROF. S. J., Univ. of California, Berkeley, Calif.

ISAACS, MR. RAPHAEL, Univ. of Cincinnati, Cincinnati, Ohio.

ISELEY, PROF. F. B., Central College, Fayette, Mo.

Jackson, Prof. C. M., Univ. of Missouri, Columbia, Mo.
Jacobs, Mr. Murkel H., Univ. of Pennsylvania, Zool. Lab., Philadelphia.
Jennings, Prof. H. S., Johns Hopkins University, Baltimore, Md.
Jenner, Prof. E. A., Simpson College, Indianola, Iowa.
Jones, Prof. Lynds, Oberlin College, Oberlin, Ohio.
Just, Prof. E. E., Howard University, Washington, D. C.

Kelley, Mr. F. J., University of Wisconsin, Madison, Wis.
Kellicott, Prof. W. E., Goucher College, Baltimore, Md.
Kelly, Mr. J. P., 2163 Gleason Avenue, Unionport, New York.
Kennedy, Dr. Harris, Readville, Mass.
King, Dr. Helen Dean, Wistar Institute, Philadelphia, Pa.
Kingsbury, Prof. B. F., Cornell Univ., Ithaca, New York.
Kingsley, Prof. J. S., Univ. of Illinois, Urbana, Ill.
Kirkham, Dr. W. B., Yale University, New Haven, Conn.
Knight, Miss Marian V., Smith College, Northampton, Mass.
Knower, Prof. H. McE., Univ. of Cincinnati, Cincinnati, Ohio.
Knowlton, Prof. F. P., Syracuse University, Syracuse, New York.
Knudson, Prof. Lewis, Cornell University, Ithaca, N. Y.
Kribs, Dr. Herbert, Univ. of Pennsylvania, Philadelphia, Pa.

Lambert, Dr. R. A., Columbia Univ., Dept. Pathology, New York City.
Lee, Prof. F. S., 437 West 59th Street, New York City.
Lefevre, Prof. George, Univ. of Missouri, Columbia, Mo.
Lewis, Prof. I. F., Univ. of Wisconsin, Madison, Wis.
Lewis, Mrs. W. H., 1931 East 31st St., Baltimore, Md.
Lewis, Prof. W. H., Johns Hopkins University, Baltimore, Md.
Lillie, Prof. Frank R., University of Chicago, Chicago, Ill.
Lillie, Prof. R. S., Clark University, Worcester, Mass.
Linton, Prof. Edwin, Washington and Jefferson College, Washington, Pa.
Loeb, Prof. Jacques, Rockefeller Institute, New York City.
Loeb, Dr. Leo, St. Louis Skin and Cancer Hospital, St. Louis, Mo.
Lowther, Mrs. Florence deL., Barnard College, Columbia Univ., New York.

Luscombe, Mr. W. O., Woods Hole, Mass.
Lyman, Prof. George R., Dartmouth College, Hanover, N. H.
Lynch, Miss Clara J., Smith College, Northampton, Mass.
Lyon, Dr. E. P., St. Louis Univ. Medical School, St. Louis, Mo.
Lund, Mr. E. J., Johns Hopkins University, Baltimore, Md.

McClendon, Dr. J. F., Cornell University Medical School, New York City.
McClung, Prof. C. E., Univ. of Pennsylvania, Philadelphia, Pa.
McGill, Dr. Caroline, Murray Hospital, Butte, Montana.
McGregor, Dr. J. H., Columbia University, New York City.
McIndoo, Dr. N. E., Bureau of Entomology, Washington, D. C.
Mackenzie, Prof. Mary D., Carnegie Institute Technology, Pittsburgh, Pa.
McMurrich, Prof. J. P., University of Toronto, Toronto, Canada.
Mall, Prof. F. P., Johns Hopkins University, Baltimore, Md.
Malone, Dr. E. F., Univ. of Cincinnati, Cincinnati, Ohio.
Martin, Miss Bertha E., University of Chicago, Dept. Zool., Chicago, Ill.
Marquette, Mr. William G., Columbia Univ., New York City.
Mast, Prof. S. O., Johns Hopkins Univ., Baltimore, Md.
Mathews, Prof. A. P., Univ. of Chicago, Chicago, Ill.
Mayer, Dr. A. G., Maplewood, N. J.
Meigs, Dr. E. B., Radnor, Pa.
Meltzer, Dr. S. J., 107 West 122d Street, New York City.
Metcalf, Prof. M. M., Oberlin College, Oberlin, Ohio.
Minor, Miss Marie L., Wadleigh High School, New York City.
Morgan, Prof. H. A., Agricultural Experiment Station, Knoxville, Tenn.
Moore, Prof. G. T., Missouri Botanical Gardens, St. Louis, Mo.
Moore, Prof. J. Percy, Univ. of Pennsylvania, Philadelphia, Pa.
Morrill, Prof. A. D., Hamilton College, Clinton, New York.
Morrill, Dr. C. V., New York University, New York City.
Murbach, Dr. L., Poultney, Vermont.

Nachtrieb, Prof. Henry F., Univ. of Minnesota, Minneapolis, Minn.

NEAL, PROF. H. V., Tufts College, Mass.
NEWMAN, PROF. H. H., University of Chicago, Chicago, Ill.
NICHOLS, DR. M. LOUISE, 3207 Summer Street, Philadelphia, Pa.
NOWLIN, MISS NADINE, Univ. of Kansas, Lawrence, Kans.

OLMSTED, PROF. J. M. D., Richmond College, Richmond, Va.
OSBURN, PROF. R. C., 557 West 124th Street, New York City.
OSTERHOUT, PROF. W. J. V., Harvard University, Cambridge, Mass.

PACKARD, DR. CHARLES, Columbia Univ., Dept. Zool., New York City.
PACKARD, DR. W. H., Bradley Polytechnic Institute, Peoria, Ill.
PAINTER, MR. T. S., Yale University, New Haven, Conn.
PAPPENHEIMER, DR. A. M., Columbia Univ., Dept. Pathology, New York City.
PARKER, PROF. G. H., 16 Berkeley Street, Cambridge, Mass.
PATON, DR. STEWART, Princeton Univ., Princeton, N. J.
PATTEN, MISS J. B., Elm Brook, South Natick, Mass.
PATTEN, DR. WILLIAM, Dartmouth College, Hanover, N. H.
PATTERSON, PROF. J. T., University of Texas, Austin, Texas.
PAYNE, PROF. FERNANDUS, Univ. of Indiana, Bloomington, Ind.
PEARSE, PROF. A. S., Univ. of Wisconsin, Madison, Wis.
PIKE, PROF. FRANK H., 437 West 59th St., New York City.
PRENTISS, MISS HENRIETTA, Normal College, New York City.

QUACKENBUSH, MR. L. S., 27 West 73d Street, New York City.

RANKIN, PROF. W. M., Princeton Univ., Princeton, N. J.
REA, DR. PAUL M., Charleston Museum, Charleston, S. C.
REIGHARD, PROF. JACOB, Univ. of Michigan, Ann Arbor, Mich.
REINKE, MR. E. E., Princeton Univ., Princeton, N. J.
RICE, PROF. EDWARD L., Wesleyan Univ., Delaware, Ohio.
ROBBINS, MR. W. J., Cornell Univ., Ithaca, New York.
ROBERTS, MISS EDITH A., Mount Holyoke College, South Hadley, Mass.
ROBERTSON, PROF. W. R. B., Univ. of Kansas, Lawrence, Kans.
ROGERS, PROF. CHARLES G., Syracuse Univ., Syracuse, N. Y.
ROSENOW, DR. E. C., People's Gas Building, Chicago, Ill.
RUDDIMAN, MISS MARGUERITE, 441 Senator Street, Brooklyn, New York.

SANDS, MISS ADELAIDE G., 348 N. Main St., Port Chester, New York.
SANDS, DR. GEORGIANA, 348 N. Main Street, Port Chester, New York.
SCOTT, PROF. G. G., College of the City of New York, New York City.
SCOTT, PROF. JOHN W., University of Wyoming, Laramie, Wyo.
SHOREY, DR. MARIAN L., Milwaukee-Downer College, Milwaukee, Wis.
SHULL, DR. A. FRANKLIN, University of Michigan, Ann Arbor, MICH.
SMITH, DR. BERTRAM G., State Normal College, Ypsilanti, Mich.
SOLLMAN, DR. TORALD, Western Reserve University, Cleveland, Ohio.
SPAULDING, PROF. E. G., Princeton Univ., Princeton, N. J.
SPENCER, DR. H. J., 1124 Amsterdam Avenue, New York City.
STEWART, MISS MARY W., Barnard College, Columbia Univ., New York City.
STOCKARD, PROF. C. R., Cornell Medical College, New York City.
STREETER, DR. GEORGE L., University of Michigan, Ann Arbor, Mich.
STRONG, DR. O. S., 437 West 59th St., New York City.
STRONG, DR. R. M., University of Chicago, Chicago, Ill.
STURTEVANT, MR. A. H., Columbia Univ., Dept. Zool., New York City.
SUMNER, DR. F. B., Sausalito, Calif., Albatross.

TASHIRO, DR. SHIRO, University of Chicago, Chicago, Ill.
TAYLOR, MISS KATHARINE A., 1304 Eutaw Place, Baltimore, Md.
TENNENT, PROF. D. H., Bryn Mawr College, Pa.
TERRY, PROF. O. P., Indiana Medical School, W. Lafayette, Ind.
THOMAS, DR. ADRIAN, 2517 Hanover Avenue, Richmond, Va.
THOMPSON, PROF. CAROLINE B., 195 Weston Road, Wellesley, Mass.
TINKHAM, MISS FLORENCE L., 56 Temple Street, Springfield, Mass.
TOMPKINS, MISS ELIZABETH M., 2019 Bedford Avenue, Brooklyn, N. Y.

TREADWELL, PROF. A. L., Vassar College, Poughkeepsie, N. Y.
TURNER, MR. C. L., Ohio Wesleyan Univ., Delaware, Ohio.

USHER, MISS SASANNAH, 1007 West Illinois St., Urbana, Ill.

VAN CLEAVE, DR. H. J., University of Illinois, Urbana, Ill.
VAUGHAN, DR. T. C., U. S. Geological Survey, Washington, D. C.

WAITE, PROF. F. C., Western Reserve Univ. Medical School, Cleveland, O.
WALKER, DR. GEORGE, Charles & Center Streets, Baltimore, Md.
WALLACE, PROF. LOUISE B., Mt. Holyoke College, South Hadley, Mass.
WARD, PROF. H. B., Univ. of Illinois, Urbana, Ill.
WARREN, PROF. HOWARD C., Princeton Univ., Princeton, N. J.
WASTENEYS, MR. HARDOLPH, Rockefeller Institute, New York City.
WATSON, MR. FRANK E., 218 Washington Street, Geneva, New York.
WATSON, PROF. J. B., Johns Hopkins University, Baltimore, Md.
Wheeler, PROF. W. M., Bussey Institution, Forest Hills, Boston, Mass.
WHEELER, MISS ISABEL, The Hattersley, Toledo, Ohio.
WHERRY, DR. W. B., Cincinnati Hospital, Cincinnati, Ohio.
WHITNEY, DR. DAVID D., Wesleyan Univ., Middletown, Conn.
WERBER, DR. E. I., 1504 E. 62d Street, Chicago, Ill.
WIEMAN, PROF. H. L., Univ. of Cincinnati, Cincinnati, Ohio.
WILCOX, DR. ALICE W., 56 Alumni Avenue, Providence, R. I.
WILDMAN, DR. E. E., 4331 Osage Avenue, Philadelphia, Pa.
WILLIAMS, DR. ANNA W., 549 Riverside Drive, New York City.
WILSON, PROF. H. V., University of North Carolina, Chapel Hill, N. C.
WOGLOM, DR. WILLIAM H., Columbia University, New York City.
WOLFE, PROF. JAMES J., Trinity College, Durham, N. C.
WOODRUFF, PROF. L. L., Yale University, New Haven, Conn.
WRIGHT, PROF. R. RAMSAY, University of Toronto, Toronto, Canada.

YERKES, PROF. R. M., Harvard University, Cambridge, Mass.
YOUNG, MR. D. B., Amherst College, Amherst, Mass.

THE MIGRATION OF THE GERM CELLS IN AMIURUS NEBULOSUS.

FREDA M. BACHMANN.

A study of the germ cells in *Amiurus nebulosus* was undertaken with the hope of determining their origin, but because of the difficulties in distinguishing them in the younger stages of the embryo and the limited amount of time in which this work was done, it was necessary to limit the subject to a study of their number and migration after they are clearly distinguishable.

The material was fixed in either Zenker's fluid or bichromate acetic. The two fluids appear to give equally good results. All sections were cut 10 microns thick and stained with Heidenhain's iron-alum hematoxylin followed by Congo red or with Mayer's hæmalum followed by Orange G.

The origin and migration of the sex cells and the formation of the germ gland in teleosts have been studied in a number of species by several investigators. Some have concluded that the germ cells are all or in part transformed epithelial cells; others believe they are never a part of the body tissues but are derived from undifferentiated cells in the very early stages of development and later migrate into the sex gland where by division, they give rise to oogonia and spermatogonia.

Nussbaum ('80) in working on the trout, found no evidence of any epithelial cells transformed into germ cells. At a certain stage in development, the germ cells divide and form little groups or nests of cells. He thought these could not be transformed epithelial cells because if the cells in the groups are of epithelial origin, then why should they be in groups, and also, if epithelial cells are continually being transformed into germ cells, then why, when these smaller cells appear in groups, do the larger cells which were in stages immediately preceding so suddenly and completely disappear? Also he found if there was a large number of cells in a nest the individual cells were smaller than those in nests where there were but a few cells. He thought the size of the individual cells in a nest in proportion to the size of the nest, good proof that no epithelial cells are transformed into germ cells. The nests of cells are at first some distance apart and become nearer together as the cells increase in number by division —not as new cells are formed from epithelial cells.

MacLeod ('81) in *Hippocampus* and *Belone acus* observed that the sex cells appear in the somatopleure and splanchopleure rather late in development and thought them to be differentiated peritoneal cells. He thought the genital fold originates in a group of germ cells on the surface of the epithelium.

Hoffmann ('86) studied salmon embryos. According to him the genital glands have their origin in the primary germ cells which are first found in the median and dorsal part of the mesentery and laterally to the body wall. He could see nothing more in these germ cells than transformed and highly differentiated peritoneal cells which in the youngest stages of development increase in number by mitosis. Later these cells come to lie laterally from the mesentery and extend into the body cavity as a small fold. The cells increase in number by division and transformation of more peritoneal cells. Nests of germ cells are thus formed. Later each cell becomes surrounded by follicle cells. He could not say whether these follicle cells are connective tissue cells or of epithelial origin, *i. e.*, from a germinal epithelium. However, he inclined to the latter because the follicle cells appear before a connective tissue stroma is differentiated.

Böhi ('04) worked on the trout and the salmon. In the trout, twenty-five days after fertilization and six days later in the salmon, he found large cells among the larger epithelial cells in both the somatopleure and splanchopleure. The cells have very large nuclei but no nucleoles. The cytoplasm stains lightly and is without definite structure. The nuclear content is granular and hence takes little of the stain. The cells are more oval or ovoid in younger embryos and rounder in older ones. There is a diminution in volume before division begins. There is a definite relation between the size of the nucleus and the size of the cell—the large cells always have the larger nuclei. In two trout embryos of twenty-five days he found in the lateral plate four and six germ cells respectively. In salmon embryos of thirty-one days he found germ cells in the same position. After the cœlome is formed he found the cells in both somatopleure and splanchnopleure—in one embryo 13 in the somatopleure, 4 in the splanchnopleure and 3 between these layers.

In salmon embryos the number of germ cells remains constant up to 185 days after fertilization. But this number varies from 20 to 54. In an embryo of 154 days he found 46 germ cells, in one of 185 days, 234 cells, in one of 199 days, 373 cells. The most posterior germ cell is six or seven somites anterior to the anus. As the embryo develops the nuclei enlarge to some extent so that the ratio of the diameter of the nucleus to that of the cell becomes somewhat greater. The nucleus has a sharper contour, loses its granular appearance and shows small chromatin bodies. From 46 to 50 days after fertilization the first small nucleoles appear, later they are larger, stain more deeply and increase in number.

The genital ridge arises as a discontinuous thickening of the cœlomic epithelium in the region of a germ cell. The thickenings unite and others are formed anteriorly and posteriorly. The uniting of these thickenings takes place at about 60 days. By 82 days the ridge is continuous in the gonal part of the embryo and is represented in the progonal and epigonal region by separate thickenings. The end of the genital ridge is posterior to the anus in one salmon embryo. The genital fold rises discontinuously. The cœlome cells of the genital ridge pull apart and leave a space in which there is a fluid. This fold extends progonally and epigonally and ends in the genital ridge.

In agreement with Nussbaum and Jungersen, Böhi found the genital fold to be composed of epithelial cells and not as MacLeod had observed it—a group of germ cells on the surface of the epithelium. Böhi believed the cœlome cells to give rise to (*a*) indifferent cells, (*b*) follicle cells, (*c*) germ cells. The cells on the sides of the genital fold gradually become transformed into germ cells. This changing of epithelial cells into germ cells lasts for only a short time and in embryos of 277 days no more transition cells can be found. The epithelial cells next to the germ cells are converted into follicle cells by pressure of the germ cells against them. According to Böhi some of the germ cells are differentiated early in development while others arise from epithelial cells after the genital fold is formed.

Eigenmann ('92) in studying *Micrometrus aggregatus* found that the sex cells first become conspicuous in the mesoblast at a stage before any protovertebræ are formed. They can be seen in earlier stages but do not stand out so prominently from the other cells. Judging from their size they are probably segmentation cells of the fifth generation. The sex cells can first be distinguished about the time the blastopore closes. The earliest ones are before the mesoderm is split off from the entoderm. They differ from the surrounding cells in having well-defined rounded outlines and in the distribution of the chromatin in the nucleus. In the sex cells the chromatin is uniformly distributed in small granules; in the surrounding cells it is collected in two or three masses. Not all the eggs of this stage show the sex cells equally well. In some eggs in which the blastopore is closing none can be made out with certainty. In some eggs many large cells were seen in the ectoderm but the inner cells of the blastoderm segment more rapidly toward the close of segmentation than the outer cells and these outer ones become distended through intracellular digestion of the surrounding ovarian fluid. But it is still possible that the sex cells arise in the ectoderm. If they are segregated as early as the fifth generation, *i. e.*, before there is any differentiation into ectoderm and entoderm, it seems to be of no great importance whether at the time of the separation of the blastoderm into ectoderm and entoderm the cells lie in its inner or outer portion. There is no change in number or size up to the time the larvæ are 2.5 mm. long. The length over which the majority of cells is distributed is about 0.20 mm.

Eigenmann ('96) continued his observations on the sex cells in *Micrometrus aggregatus*. He finds that the striking feature is the asymmetry of the two sides and the variation in number and position of the cells in different larvæ. He finds the nuclei in the sex cells of embryos 5 mm. in length have become somewhat larger and the nucleoles also so that in the period of apparent inactivity there have been histogenic changes just as truly as the changes in the somatic cells although there is no division. In later stages the tissues containing the sex cells form a median ridge in the dorsal end of the mesentery. This ridge divides anteriorly into two ridges each of which end in a single little fold of the peritoneum which is differentiated only by being rich in nuclei. In some larvæ at this time the germ cells have not increased in number, in others they may have divided once. In one larva in which a few cells were widely separated from the majority, the ridge entirely disappeared to reappear in the neighborhood of some widely separated anterior cells. From such instances it is evident that the germinal ridge develops only under the influence of the reproductive cells.

Dodds ('10) in *Lophius* was able to recognize germ cells when the blastoderm had not quite half covered the yolk. At this time they are in the primary entoblast. They pass into the mesoblast when this is separated and into that part which becomes the myotome. Later when the mesoblast is separated into myotome and lateral plate the germ cells migrate into the latter and again when this splits they are left in the splanchnopleure. Later they migrate toward the median border of the cœlome and thence upward into the somatic layer and to the position of the permanent germ cells.

The number of germ cells varies but is small—not over fifty-five. From the time they are recognizable until they are in the position of the germ gland there is no increase in number. The apparent increase in early stages is due to changes in the cells which make them more easily recognizable. The cells are characterized by rounded outlines or they may be amœboid. The cytoplasm stains more deeply than that of the somatic cells, the nucleus is smaller, irregular in shape, apparently less turgid and contains two small nucleoles. The nuclei of the somatic cells have two large nucleoles or a single very large nucleole. The decrease in the size of the germ cell nucleoles is due to a loss of part of their content through the nuclear membrane into the cytoplasm. This extrusion of nuclear material does not take place simultaneously from the nucleoles of any one nucleus nor from all germ cells.

There is no segmental arrangement of the germ cells. There is both an active migration of the cells and a passive change due to growth of the surrounding tissue. Dodds is of the opinion that before any differences between germ cells and somatic cells can be detected there must be an unseen physiological difference which determines the future behavior of the cells.

The eggs of *Amiurus nebulosus* vary in size in the different nests. There is often a difference of 1 mm. in diameter. For this reason it follows that embryos of the same length are not necessarily of the same stage of development. Embryos 3.2 mm. long taken from one nest may be slightly further developed than those 3.7 mm. long taken from another nest.

When stained with iron hematoxylin the yolk granules of the egg are black but if this is washed out with iron alum and then followed by congo red the granules are a deep red. The yolk material in the germ cells is more or less diffused through the cytoplasm and loses the black hematoxylin stain quite readily in the iron alum. But because of the contained yolk the cytoplasm of the germ cells stains a deeper red than the cytoplasm of the surrounding cells. Occasionally there is a small compact mass of yolk in a germ cell which retains the black stain. Yolk granules of the egg stain lightly with Mayer's hæmalum but take up the Orange G very rapidly. Hence the cytoplasm of the germ cells stains a light orange with hæmalum and Orange G. The cytoplasm of the surrounding cells is a bluish gray.

The germ cells in all of the stages studied up to the time of multiplication were found to be about the same size. They are from 14 to 18 microns in diameter and the nuclei 7 to 9 microns in diameter. The germ cells are quite well rounded though sometimes they are more or less amœboid. The cytoplasm is quite clear, especially so if the contained yolk is in one or two compact masses. The nucleus is spherical or frequently lobed. But this lobed condition of the nucleus is not peculiar to the nuclei of germ cells—it is often found in nuclei of other cells. There are often two nucleoles but again this is no peculiarity of germ cell nuclei. In the very early stages of development most of the somatic cells have more than one nucleole and two nucleoles are seen in the nuclei of many somatic cells long after the cœlomic cavity is formed. Not infrequently there are no nucleoles in the germ cell nuclei. The chromatin which is fairly distinct is scattered through the nucleus in the form of fine granules. It is never aggregated into large masses as is common in the nuclei of somatic cells.

The germ cells with few exceptions have been found in the mesoderm. These exceptions were a few cells in the cœlomic cavity or between the lateral plate of mesoderm and the yolk, also a few doubtful cells in the entoderm. In younger embryos the germ cells are in the lateral plate of the mesoderm and near the region where the blastopore has closed. In older embryos where the tail has grown out beyond the yolk they are in the splanchnopleure surrounding the hind gut. Later they are in the mesentery and in the germ gland anlage. The exact location of the germ cells will be given in greater detail in the description of the different stages studied.

The number of germ cells is constant until after or about the time they are in the germ gland anlage. The average number is about 23 with extremes 12 and 34. Because of the large size of the germ cells and the thickness of the sections it would be possible to count the same cells twice. However, by noting the position and appearance of the cells and the size of the nucleus, it is believed that few errors have been made in counting.

There is probably no one characteristic peculiar to germ cells in *Amiurus nebulosus*. Blood corpuscles are frequently nearly spherical. But in these the cytoplasm stains more lightly with congo red and Orange G., the nucleoles are larger, and the nuclear membrane much more distinct than in the germ cells. Also the ratio of the diameter of the nucleus to the diameter of the cell is greater in blood corpuscles than the same ratio in germ cells. In a number of blood corpuscles this ratio was 2 : 3, in germ cells it is 1 : 2. The cytoplasm of the entoderm cells often contains much yolk and hence these frequently stain like germ cells. But the entoderm cells are usually smaller and columnar or irregular in shape, and the nuclei are more often oval than spherical with two or three large nucleoles. In the earliest stage studied the mesoderm cells were not more than half the size of the germ cells and contained little if any yolk. Even in this stage the nuclei of the mesoderm cells are becoming oval or elongated. In later stages the mesoderm cells are small, very irregular in shape with cytoplasmic processes. The germ cells are easily recognized in any stage by their shape, size, and staining qualities.

EMBRYOS 3.2–3.7 MM. LONG.

In embryos of this length the blastopore is closed and the tail extends about 1 mm. beyond the yolk. The pronephric ducts have been formed. There is no trace of a split in the mesoderm which is to give rise to the cœlomic cavity. The axial mesoderm is a compact mass of cells extending laterally and ventrally and composed of cells which are still quite regular in outline although the nuclei are more or less oval. There is scarcely a trace of yolk in these mesodermal cells. The entoderm cells frequently contain large yolk masses or have yolk material diffused throughout the cytoplasm.

The germ cells are found in the lateral plate of mesoderm in the region just anterior to where the tail leaves the yolk. The germ cells may be anywhere in the lateral plate but the majority of them were found some distance away from the mid-line (Fig. 1). Not infrequently they are far out in the lateral plate as shown in Fig. 2. A few germ cells were found between the yolk and the mesoderm. It may be possible that they came from the yolk but at least none were seen in the yolk nor coming out of it. The germ cells are so very different in appearance from the other somatic cells that there is no danger in overlooking them in well-stained sections. The length of the region in which they are found varies from 0.025 mm. to 0.054 mm. The embryos are not symmetrical with regard to the number of germ cells found on each side. The total number of an embryo is from 12 to 28. The following table shows the number found on each side, the total number, and the distance throughout which the germ cells were distributed in several of the embryos sectioned.

	Right.	Left.	Total.	Length of Region, Mm.
1.....	15	8	23	0.250
2.....	8	14	22	0.260
3.....	10	9	19	0.360
4.....	14	13	28	0.250
5.....	4	9	13	0.340
6.....	12	13	26	0.640

In number 4, one cell was in the entoderm, and in number 6, a germ cell was between the yolk and the mesoderm.

Embryos 5 mm. Long.

The cœlomic cavity is completely formed. With the growth in length a greater differentiation in tissues has been going on. The germ cells are found in the region of the hind gut, the majority of them are posterior to where the tail leaves the yolk. The most anterior cells are 0.080 to 0.220 mm. anterior to this region. The most posterior cells were 0.180 to 0.290 mm. anterior to the anus. With few exceptions the germ cells were found in the splanchnopleure (Fig. 3). In one embryo a nest of four cells was found in the somatopleure (Fig. 4). In the same embryo a germ cell was found lying in the center of the cavity between the somatopleure and the ectoderm. This was such an unusual position that it seems reasonable to think that it might have been torn away from the somatopleure in sectioning; however, the section has not the appearance of having been torn in any way. The size and appearance of the germ cells of embryos 5 mm. long are the same as in the preceding stage, but they are much more conspicuous due to the greater differentiation of the surrounding mesoderm. The cells are in the dorsal half of the splanchnopleure and often give the appearance of crowding toward the mesentery next to the alimentary tract (Fig. 5). The germ cells are distributed throughout a distance averaging 0.420 mm. in length. The following table shows in three embryos the unsymmetrical arrangement, the number of cells, the length of region throughout which the germ cells are distributed, the distance anterior to the anus of the most posterior germ cell and the distance anterior to the region where the tail leaves the yolk of the most anterior germ cell. In embryo number 2, 2 germ cells were in the mesentery; 6 cells were in the mesentery in number 3.

	R.	L.	Total.	Length of Germ Cell Region, mm.	Dis. Ant. to Anus, mm.	Dis. Ant. to Tail, mm.
1.	6	26	32	0.420	0.100	0.230
2.	6	17	25	0.450	0.180	0.100
3.	9	7	22	0.400	0.290	0.220

EMBRYOS 7 MM. LONG.

Three embryos of this length were sectioned. In one of these there were 26 germ cells most of which were in the mesentery or at least very near it (Fig. 6). In the other two embryos the germ cells had probably divided once although there was little difference in the size of the cells from those in earlier stages. The cells were 14–16 microns in diameter and the nuclei 7–8 microns. There were 57 cells in one embryo and 63 in the other. In both embryos most of the germ cells were crowded in the mesentery in nests of 3 to 8 (Fig. 7). A few single cells were nearer the germ gland anlage or still in the splanchnopleure. Except in the case of one cell dividing, the appearance of the nuclei, cytoplasm, etc., seemed the same as in cells in earlier stages. The germ cells are in the same region as in the 5 mm. stage. The following table gives the number of cells, the length throughout which the cells were distributed, the distance anterior to the anus of the most posterior cell, and the distance anterior to the tail region of the most anterior cell.

	Total No.	Length of Region, Mm.	Dis. from Anus, Mm.	Dis. from Tail, Mm.
1.	26	0.680	0.120	0.220
2.	63	0.400	0.210	0.240
3.	57	0.430	0.120	0.320

EMBRYOS 9 MM. LONG.

In embryos of this length the anlagen of the germ glands appear as a fold in the peritoneum on either side of the base of the mesentery. (Figs. 8 and 9). This fold extends from 0.030 to 0.280 mm. anterior to the most anterior germ cell. In one embryo the fold was poorly developed for a distance of 0.080 mm. where there were no germ cells but this is not the most common condition. After the fold once appears, it is continuous throughout the region in which the germ cells are found. In one embryo there were no germ cells for a distance of 0.200 mm. but in this entire distance the fold of the peritoneum was very prominent. The fold seems to extend as far posterior from the germ cell region. It was found to extend 0.080 to 0.230 mm. posterior from the most posterior cell. In all of the embryos of this stage which were sectioned the germ cells had found their way into the peritoneal fold. The number remains the same—28 to 30. The size and appearance of the cells agree exactly with the same in the earliest stages. The germ cells are distributed throughout a distance of 0.980 to 1.020 mm. The most posterior cells are 0.420 to 0.520 mm. anterior to the anus. It will be seen that although the embryos have increased much in size, the length throughout which the germ cells are distributed in proportion to the length of the embryo is about the same.

Embryos 14 mm. Long.

In all of the embryos of this stage the germ cells had divided at least twice. There had probably been two divisions in one embryo where the number was 144. Here the germ cells measure 8–12 microns in diameter and the nuclei are 4–6 microns. The chromatin is no longer in such fine granules as in the early stages but is often aggregated into small masses. The germ cells are in the center in the peritoneal fold with strands of peritoneal cells between them (Fig. 10). There is nothing which would suggest a differentiation of peritoneal cells into germ cells. Some of the germ cells are smaller than others but it is believed that these are cells which have divided more times than the larger ones and not that they are transition cells (see Fig. 10). No germ cells were found as large as even the smallest of those in younger stages. The germ cells and peritoneal cells stain as distinctly different as described before and no cells intermediate in size or staining qualities were found. In this embryo of 144 germ cells the germ gland or fold extends only 0.010 mm. anterior to the first germ cell but 0.500 mm. posterior from the most posterior germ cell. The germ cells were distributed throughout a distance of 1.280 mm. and the germinal fold throughout 1.790 mm.

In another embryo of the same length the germ cells had evidently divided several times—the cells here were only 6 or 7 microns in diameter and the nuclei about 34 in diameter. There are twenty or more germ cells in every section of each of the glands. The cells were not counted in the entire embryo. They are distributed throughout a distance of 1.220 mm. The most posterior germ cell is 0.040 mm. posterior to the anus and the germ gland extends 0.160 mm. posterior to the anus. The germinal fold extends only 0.050 mm. anterior to the first germ cell.

Conclusions.

Germ cells in *Amiurus nebulosus* are distinct from all other cells at least from the time the embryo is 3.2 mm. long. They are then in the lateral plate of the mesoderm. The number of germ cells varies in the different embryos. Until the time when these cells are in or near the germ gland anlage, the average number is 23. With respect to the number of germ cells found on each side the embryo is not symmetrical. When the cœlome is formed they are largely in the splanchnopleure in which they migrate toward the mesentery and in this to the germ gland anlage. There is in most cases no multiplication in number of cells up to the time the germinal fold is formed. The cells migrate into this fold and then divide. There was no transition of peritoneal cells into germ cells observed in any of the stages studied. The epithelial covering and the stroma of the germ gland is derived from peritoneal cells. The germinal fold develops in regions where there are no germ cells.

This work was done at the university of Wisconsin under the direction of Prof. B. M. Allen, to whom the writer is greatly indebted for the material and for helpful advice and suggestions.

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EXPLANATIONS OF THE FIGURES.

All figures were outlined with the aid of a camera lucida.

PLATE I.

FIGS. 1 AND 2. Germ cells in lateral part of mesoderm. × 390.

FIG. 3. Formation of cœlome. Germ cells in splanchnic layer. × 390.

FIG. 4. A nest of germ cells in somatopleure. × 390.

FIG. 5. Germ cells migrating toward mesentery. × 390.

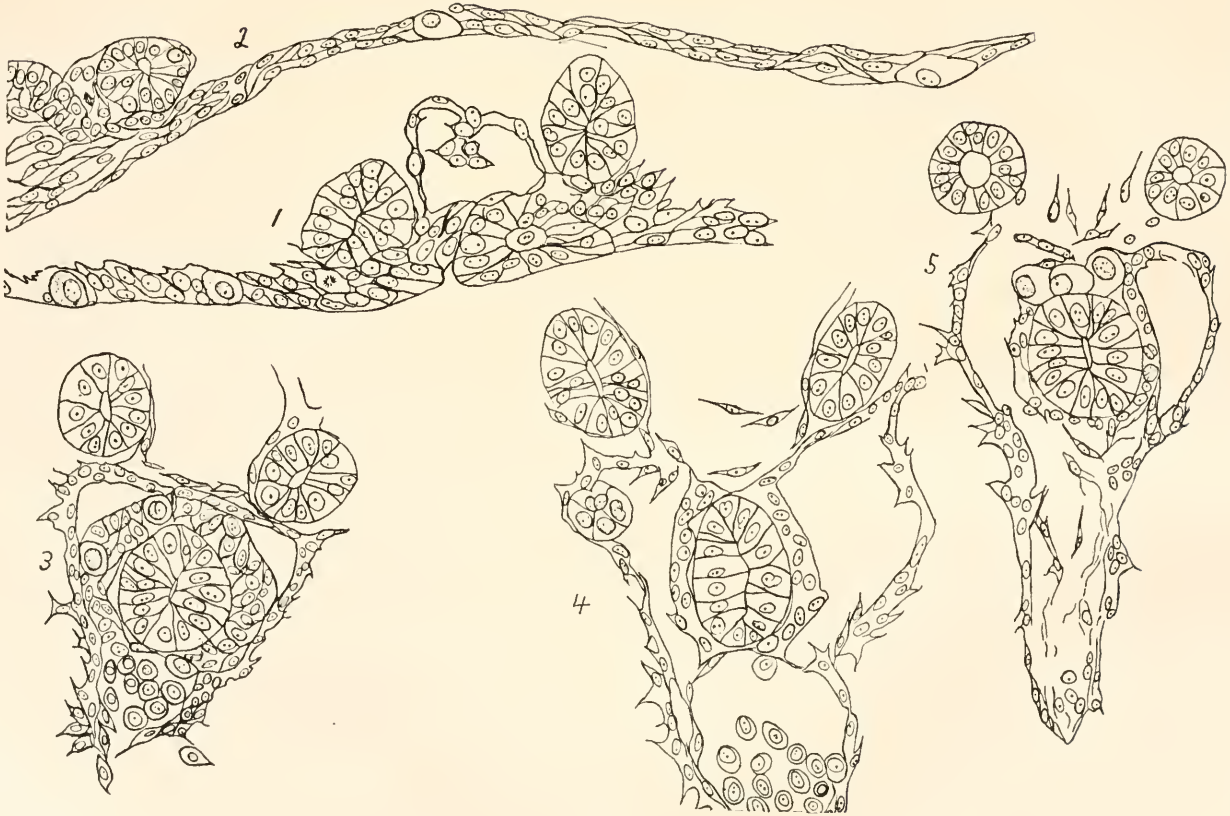

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PLATE II.

FIGS. 6 AND 7. Germ cells migrating dorsally in mesentery. × 390.

FIG. 8. Diagrammatic representation of cross section of embryo to show the relative position of the germ glands. × 72.

FIG. 9. The origin of the germ gland from a fold in the peritoneum. × 390.

FIG. 10. Germ cells within the germ glands. × 390.

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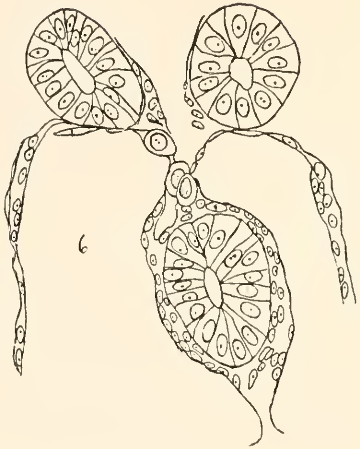

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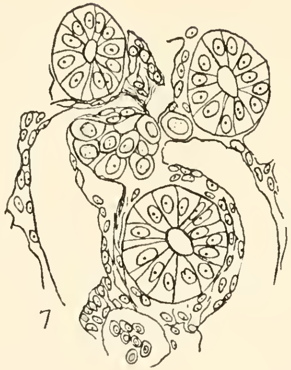

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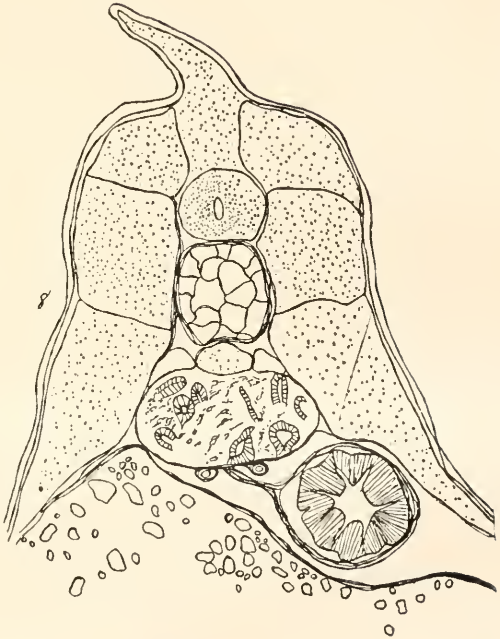

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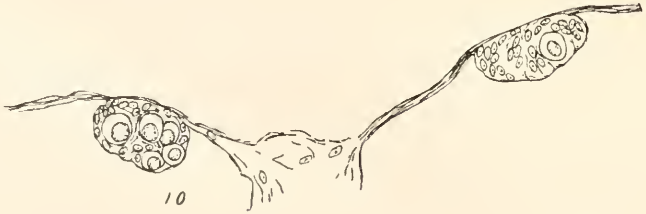

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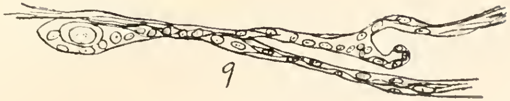

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A QUALITATIVE ANALYSIS OF THE EGG-SECRETIONS AND EXTRACTS OF ARBACIA AND ASTERIAS.

OTTO GLASER.

(From the Marine Biological Laboratory at Woods Hole and the Zoölogical Laboratory of the University of Michigan.)

INTRODUCTION.

Although it must have been seen earlier, F. R. Lillie ('12) was the first, so far as I know, to throw emphasis on the observation that when unfertilized *Arbacia* eggs are allowed to stand in a small quantity of sea water, the supernatant liquid takes on a reddish-brown color. The amount of discoloration varies directly as the number of eggs present, the amount of sea water used, and within limits, the time of exposure. If the eggs are now precipitated with the centrifuge, and the fluid above is decanted and filtered through paper, or simply decanted without filtration, it can be very easily shown that substances are present which do not occur in ordinary sea water.

I. ANALYSIS BY MEANS OF SPERM–SUSPENSIONS.

The best method of analysis is that discovered by F. R. Lillie, and although it was not my primary purpose, I have verified a number of his results ('12[1], '13[1], '13[2]). Following Lillie, I shall present my material under four heads: (*A*) Activation; (*B*) Chemotaxis; (*C*) Agglutination; (*D*) Paralysis.

A. Activation.

1. *Iso-Activation.*—As Lillie has pointed out, the phenomena of activation are presented with unequal clearness in different forms. The spermatozoa of *Nereis* for instance are so active in sea water that the effect of the egg-secretion is obscured. With *Arbacia*, the sperm, as is well known, are also active in sea-water, nevertheless a noticeable increase in the rate of their movements can be observed after the addition of a drop of the egg-secretion. *Asterias forbesii* is really much more favorable than either *Nereis* or *Arbacia* for the detection of the activating effect, for here the

spermatozoa are almost motionless. The suspensions I worked with showed only isolated spermatozoa executing occasional spasmodic movements. Upon the addition of the secretion from its corresponding egg, the *Asterias* sperm are thrown into violent activity contrasting sharply with their original state of quiescence.

2. *Hetero-Activation.*—Essentially the same phenomenon described under the head of iso-activation can be observed if *Asterias* sperm are treated with *Arbacia* secretion, and *Arbacia* sperm with that from *Asterias* eggs. As might be expected the phenomenon is more marked with the *Asterias* sperm on account of their original inactivity, although the *Arbacia* sperm are also noticeably accelerated. I have made no experiments capable of deciding whether the activation of the two kinds of spermatozoa is due to the existence in the secretions from the two kinds of eggs of the same substance or of specifically different substances.

3. *Re-Activation.*—Activation is a temporary state, and after certain other reactions have occurred, the spermatozoa are found to be quite immotile. Such spermatozoa, although chemically different (13[1]) from fresh ones which have never been subjected to the secretions, are nevertheless capable of re-activation. This is shown by the following experiments, in which *Arbacia* sperm-suspensions prepared in each case from a single male were divided into lots, activated by the addition of secretion, observed at intervals, and treated with more secretion as well as fresh eggs. The degrees of activation, reactivation, or movement are given as great, moderate, slight, or zero. In the instances in which eggs were added, fertilizations always took place, but the proportion of eggs that divided varied inversely with the length of exposure of the sperm to the secretion (p. 369).

B. CHEMOTAXIS.

The chemotactic effect of the egg-secretion has been studied very carefully by Lillie, and both methods and results have been described by him at length ('13[1]). I have verified the most essential results on *Arbacia* and have extended them to *Asterias*. The injected-drop method as well as the distribution of sperm about groups of eggs were used as indicators. As Lillie suggests, such results do not make clear the rôle of chemotaxis in normal

ARBACIA SPERM.

Experiment 1.

Treatment.	Lot A, Activation.	Lot A_1, Activation.	Lot A_2, Activation.	Lot A_3, Activation.	Lot A_4, Activation.	Time.
Added secretion	Strong	Strong	Strong	Strong	Strong	11.05 A.M.
	Moderate	Moderate	Moderate	Moderate	Moderate	11.10
Added secretion	....	Strong	....	....	....	11.10
Added eggs....	....	....	Strong	....	....	11.10
	....	Slight	Slight	....	....	11.11
	Zero	....	....	Zero	Zero	3.30 P.M.
Added secretion	....	....	....	Slight	....	3.30
Added eggs....	....	....	....	....	Slight	3.30

Experiment 2.

Added secretion	Strong	Strong	Strong	....	....	12.10 P.M.
	Slight	Slight	Slight	....	....	1.10
Added secretion	....	Moderate	....	....	....	1.10
Added eggs....	....	....	Moderate	....	....	1.10

Experiment 3.

Added secretion	Strong	Strong	Strong	Strong	Strong	12.00 M.
	Slight	Slight	Slight	Slight	Slight	1.00 P.M.
Added secretion	....	Moderate	....	....	....	1.00
Added eggs....	....	....	Moderate	....	....	1.00
	....	....	....	Zero	Zero	5.00
Added secretion	....	....	....	Slight	....	5.00
Added eggs....	....	....	....	....	Slight	5.00

fertilization. One of the difficulties is the thigmotactic response of the sperm, as this insures their sticking to surfaces they may chance to meet. It is easy to see how under such circumstances an accumulation about eggs might take place. While not conclusive, experiments in which the surface of the eggs was very materially reduced, and the jelly mass quite obliterated by desiccation, showed that even in this case the sperm collect in great numbers about the eggs. Minute fragments of egg-powder are also centers about which the sperm aggregate in great numbers. I have not observed this when powdered glass or sand were added to the suspensions and as the surfaces in these cases although of different physico-chemical properties, must be assumed to be at least as extensive as in the egg-powder, the marked sperm aggregation about the dried egg-fragments and its absence about the other fragments is certainly in harmony with the idea that chemotaxis is a factor in bringing the sperm to the egg.

C. Agglutination.

1. *Iso-agglutination.*—That egg-secretion is capable of agglutinating sperm has been fully described by Lillie ('12^1, '13^1, '13^2). In this section I simply wish to add my testimony to his as to the facts in the case—namely that this phenomenon occurs; that it is reversible; that its duration is brief and may be utilized as a measure of concentration; and that sperm once agglutinated, although capable of re-activation, do not agglutinate again.

2. *Hetero-agglutination.*—I have found that the *Arbacia* secretion also agglutinates the sperm of *Asterias*, and that *Asterias* secretion, besides agglutinating its own, has a similar effect on the spermatozoa of *Arbacia*. Thus

1. *Arbacia* sperm + *Arbacia* secretion = agglutination masses described by Lillie.
2. *Arbacia* sperm + *Asterias* secretion = dense, angular masses. Reaction slower than 1.
3. *Asterias* sperm + *Asterias* secretion = masses smaller than 1. Angular.
4. *Asterias* sperm + *Arbacia* secretion = result similar to 3.

At the time when these experiments were performed, the material had practically disappeared, and in consequence the question whether the above hetero-agglutinations are due to the same substances as the iso-agglutinations, or whether each secretion contains both an iso- as well as a hetero-agglutinin, could not be decided. Since hetero-agglutination between *Arbacia* and *Nereis* is not brought about by the same substance ('13^1) that causes the iso-agglutination, the same relations may hold for *Arbacia* and *Asterias*. It will prove interesting to see whether the agglutinin in *Arbacia* secretion that reacts with the *Nereis* sperm is the same one that agglutinates *Asterias* spermatozoa. If not, it becomes important to discover how many agglutinins are present. In this connection I may refer to an observation already published (13^3), namely that the *Arbacia* secretion agglutinates the larvæ of *Arenicola*.

3. *Origin of the Agglutinin.*—Lillie has shown that the agglutinin is chiefly located in the outer jelly of the *Arbacia* egg. I have found the same thing to hold true of the *Asterias* ovum. It was also proved by Lillie (13^2) that if the outer jelly is removed

by shaking, the eggs after two or three washings impart only a weak agglutinating power to the supernatant water. This however increases in the course of time. From this Lillie concludes that the eggs produce the agglutinating substance. Moreover he has shown that they are the only tissue of *Arbacia* that does produce this material.

My own experiments show that the agglutinating substance is located in greatest abundance in the jelly and that the eggs also contain this material. As additional evidence it may be stated that when eggs are inseminated with fairly concentrated sperm-suspensions, the collection and activity of the spermatozoa may be great enough to tear the outer jelly away from the egg. When this occurs, one may suddenly observe great balls of sperm apparently cast off from the eggs and forming huge agglutination masses. In this instance many sperm also remain in contact with the egg, which later shows a typical fertilization membrane, and divides. This point is important in connection with the mistaken idea that the outer jelly is essential for the appearance of the fertilization membrane ('13[4]).

In connection with the existence of agglutinin in the egg, I may refer to experiments with egg-powder in which very effective agglutinations were secured. It cannot well be supposed that every fragment of egg-powder has bits of dried jelly adhering to it.

4. *The Mechanism of Agglutination.*—On this point Lillie's inference based on the *Nereis* sperm, in which because of relatively great size and slow movement, direct observation is possible, can be substantiated by a variety of observations. Lillie noticed that the agglutination is between the heads, and that the tails, at least until the period of paralysis sets in, are not visibly affected. "The adhesion of the heads demonstrates some change in the membrane that renders them sticky" ('13[1], p. 556), and direct observation showed "that in agglutinated masses the heads of many of the spermatozoa are swollen into spherical form and have lost the normal strong refringibility. The change is in this case a very characteristic one indicating a great change in permeability" (*loc. cit.*).

The capacity for influencing the permeability of cells is by no means limited to the sperm. Thus, as I have pointed out ('13^3), egg-extract added to blastulæ which have developed in normal sea-water, slows their movements, and increases their volume, indicating a change in permeability. R. S. Lillie has emphasized on numerous occasions the great advantages of *Arenicola* embryos ('13^5 and earlier papers) as indicators of permeability changes which may be registered by the outflow of pigment. When the *Arbacia* secretion is added to the *Arenicola* larvæ, movement is slowed down, the pigment flows freely into the water, indicating an increase in permeability, and a slight and reversible agglutination occurs. Considering all the facts at present available, it seems reasonable to suppose that agglutination is the result of an increase in permeability, and we may imagine that the exudation of material from the cells or the changes that lead to the exudation, render them sticky. Unless some other chemical reaction is involved, it seems to me more likely that the occurrence of agglutination depends upon the exudate. The sperm of *Nereis*, the blastula of *Arbacia*, and the larvæ of *Arenicola* appear to furnish us with three out of four theoretically possible types of cases.

D. Paralysis.

The addition of egg-secretion to a sperm-suspension is followed by activation, a chemotactic effect, and a reversible agglutination. For some time after the agglutination masses have disappeared, the sperm remain quite active, but the rate of their movements decreases until finally they come to a standstill, and appear as though paralyzed. As the re-activations show, this paralysis is not an irreversible state, although the second period of activity never lasts as long as the first, nor is the activity on the whole as great while it lasts. The third activation may be almost momentary. Re-activated sperm are capable of fertilizing the eggs.

II. QUALITATIVE CHEMICAL ANALYSIS OF THE EGG-SECRETION.

Although the observations recorded are important for an understanding of the nature of the egg-secretion, they have been reported at this time and in this connection chiefly for the interest they may have in relation to the factual basis of Lillie's theory of fertilization ('13[2]). Analyses by other methods were attempted.

It is altogether likely, and in the case of the *Nereis-Arbacia* hetero-agglutination, definitely proved, that the egg-secretion contains more than one substance. The problem therefore presents itself of isolating these bodies. A first step toward orientation has been made by means of certain qualitative chemical tests.

The method of securing egg-secretion finally adopted was suggested to me personally by Dr. F. R. Lillie, and consists in adding to a certain number of "dry" eggs, double their volume of sea-water, and with occasional slight agitation, allowing ten minutes to elapse. At the end of this time the ova were precipitated by 100 revolutions of the centrifuge, and the supernatant fluid, a clear, golden liquid in the case of *Arbacia*, or whitish and opalescent in the case of *Asterias*, was usually carefully decanted without filtration through paper. Such solutions I have adopted as standard. With the *Arbacia* secretion the following tests were made:

1. The solution is gold-yellow in color and clear.
2. The solution is neutral to litmus.
3. Upon cooling to 0° no change was noticeable.
4. Upon boiling the color becomes faintly purple.
5. The purple coloring matter may be removed at least in part if white of egg is allowed to coagulate in the boiling solution.
6. No acid-insoluble precipitate is formed upon the addition of $n/10$ NaOH.
7. 1 or 2 drops conc. HCl produced faint cloudiness which became more distinct on standing.
8. The addition of alcohol produced no visible change.
9. Millon gave a white precipitate with no color change on boiling.
10. The biuret test was negative.
11. HNO_3 gave no ring but a faint cloudiness.
12. The xanthoproteic test gave no precipitate, but the solution turned distinctly yellow.
13. The Adamkiewicz test was negative.
14. Fehling gave no reduction.

15. Bi-subnitrate gave no reduction.

So far as these tests go, and they were repeated several times, it seems likely that reducing substances are absent, and that if proteins are present, their concentration is too low to give the ordinary reactions in their usual form. The opalescence observed upon the addition of acids, and the yellow color gotten in the xanthoproteic test, indicate possibly minute traces of protein but these may come from traces of the egg jelly.

With all the tests that made such experiments possible, the secretion was afterwards tested as to its agglutinating property, and was found in every case to still possess this power, tests 3, 4, and 5. No exact quantitative comparison as to the agglutinating strength before and after boiling was made. Lillie states ('13[1], p. 557) that the agglutinin when boiled and then allowed to stand at 95° for 30 minutes is destroyed in large part, and almost entirely if kept at this temperature for 66 minutes. In some of my experiments the secretion was brought to the boiling point, in others it was boiled 5 minutes. The color change noted always occurred, but the agglutination power appeared undiminished. Both of these results are described in Lillie's paper ('13[1]).

As to the nature of the purple substance, I may say that even if its formation, and presumably with that, the abstraction of something from the original solution makes no measurable difference in the agglutinating power of the secretion, this material may nevertheless be significant in other connections. My reasons for suggesting this are that when a sperm suspension is added to the secretion, traces of this purple color appear; when dilute sperm suspensions are killed by heat the same color is seen; in concentrated suspensions the red dominates over the blue; and in still more concentrated suspensions the color is like that of port wine. This same color also appears in desiccated eggs as well as sperm. From these facts the thought lies near at hand, that we are here dealing with the production of a compound specific for *Arbacia*. Corresponding experiments with *Asterias* do not give this color, nor have I gotten it with oyster sperm. On the other hand, *Asterias* sperm as well as egg-secretion turn a slight salmon-color when boiled. With the exception of these color differences, the same results in tests, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, and 14, were gotten with the *Asterias* secretion.

III. THE SECRETION OF SUBSTANCES AT FERTILIZATION AND IN HYPERTONIC SEA-WATER.

The unfertilized eggs of *Arbacia* show no noticeable decrease in volume in sea-water even four hours after removal from the ovaries ('14^1). Fertilized eggs however are measurably smaller in both *Arbacia* and *Asterias* within a few minutes after insemination ('13^3, '14^1). A change in the same sense seems also to occur in the lamprey ('14^2).

The decreased volume is probably chiefly due to the loss of water but it can be shown that in *Arbacia* other substances also leave the egg.[1]

The first thing that occurs to one in testing whether at fertilization more soluble substances are secreted than from unfertilized eggs, is to compare the agglutination strength of equal quantities of sea water, with and without sperm, to which equal quantities of unfertilized eggs have been added and allowed to remain for equal lengths of time. Such experiments show that there is more free agglutinin present in the sterile water. The reason for this is not necessarily that the eggs at fertilization secrete less of this substance, for Lillie has shown that the sperm binds the agglutinin, a fact also indicated by the experiments on re-activation without re-agglutination. Moreover it is quite possible that at fertilization other substances are secreted and combine with the agglutinin outside the egg. This alone or in conjunction with the circumstance that a portion of the agglutinin in these experiments would already be bound by the sperm in the infected sea-water would account for the finding of a smaller amount of free agglutinin by any subsequent agglutination test.

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[1] McClendon ('09) says of *Arbacia:* "When the egg is fertilized or put in 'membrane-forming' solutions a fluid is extruded which pushes the jelly out from the surface of the egg." As neither the evidence for this statement, nor any reference to the fact that something is also extruded from the unfertilized eggs, are given, I do not know whether my contention is antedated or not. McClendon also states that parthenogenetic reagents, when sufficiently concentrated cause the diffusion of pigment from the eggs into the sea-water. If these concentrations are identical with those most favorable for the initiation of cleavage, there is here an important point of identity between the artificial and normal induction of development.

Such experiments therefore are not capable of deciding whether more or less soluble substance is secreted at fertilization than before. That no agglutinin is secreted after fertilization is complete, has been demonstrated by Lillie's ('13^2) later work.

The second possibility, that of utilizing the xanthoproteic test suggested itself, for in case the yellow color were not due to traces of dissolved jelly, it might increase as the result of fertilization. As a matter of fact the yellow color gotten from sea-water over eggs inseminated as above is deeper than that given by an equal quantity of sterile water exposed to an equal quantity of unfertilized eggs for the same length of time. This was considered a promising result until it was found that the addition of even a small quantity of sperm increases the density of the color to a marked degree.

A third test however gave comparative results which are not open to this difficulty. In these experiments I made use of the fact that the unfertilized eggs of *Arbacia* discolor the sea-water. Three sets of observations were made. In each of these 1 c.c. of an egg-suspension of given concentration in which the ova were uniformly distributed, was added to each of three tubes, one of which contained 5 c.c. of sterile sea-water, the second 5 c.c. of sea-water infected with just enough spermatozoa to be very faintly opalescent, whereas the third contained 5 c.c. of "double" sea-water, *i. e.*, sea-water which had been boiled down to half its original volume. Color tests were made in the usual way, and the discoloration produced by the unfertilized eggs in a given time was taken as unity. On this basis the following results were obtained in an experiment exactly representative of the others:

	A	*B*	*C*
Time.	5 C.c. Sea-water, 1 C.c. Eggs.	5 C.c. Infected Sea-water, 1 C.c. Eggs.	5 C.c. Double Sea-water, 1 C.c. Eggs.
9.40	Color = 0	Color = 0	Color = 0
9.50	Color = 1	Color = 1.5	Color = 2
9.55	Color = 1	Color = 1.5	Color = 2
9.56	+ 1 c.c. eggs	+ 1 c.c. eggs	+ 1 c.c. eggs
10.10	Color = 2	Color = 3.0	Color = 4

The extra c.c. of eggs was added at 9.56 to see whether the first difference between *A* and *B* could be the result of either the slight opalescence due to the presence of the small amount of sperm in *B*, or to the small traces of the purple "sperm-agglutinin" compound which was necessarily formed in this tube. However the relative densities of the colors in the three tubes did not change, although the relative effect of the sperm as well as of the purple compound could not have been as great at 10.10 as at 9.50, and 9.55. The same experiment was repeated several times with more dilute suspensions of sperm, and very dense suspensions of eggs. Exactly the same relative discolorations were obtained. I mixed up the tubes on several occasions and asked some one not familiar with the experiment to see if any differences could be noted. I also had the tubes mixed and handed to me for identification. In every case it took but a moment to distinguish the tube in which fertilization had occurred. This was true also in the absence of the tube containing the double sea-water.

One may say therefore that when equal quantities of unfertilized *Arbacia* eggs are allowed to stand for equal lengths of time in equal quantities of sterile and sperm-infected sea-water, the discoloration of the supernatant liquid is greater in the case of the eggs undergoing fertilization. This proves that something in addition to water leaves the eggs at fertilization, a circumstance not at all surprising in view of the fact that, whatever else it may involve, fertilization is accompanied by an increase in permeability.[1] The question whether there is in addition to the quantitative difference, also a qualitative one, must for the present remain open.

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[1] Loeb ('13^6) criticizes McClendon's evidence for increased permeability after fertilization on the ground that more than one interpretation of the experimental evidence is possible. My contention is not that the permeability of fertilized eggs is greater than that of unfertilized, but that there is an increase in the permeability of unfertilized eggs at the moment at which they are being fertilized. This idea is expressed by R. S. Lillie, p. 290, "The Physiology of Cell Division," III. Direct observational support for this view is furnished by F. R. Lillie's work on *Nereis*, and more indirect evidence by my measurements of the rate of secretion by unfertilized eggs as compared with eggs undergoing the process of fertilization. The decreased volume of the *Arbacia* and *Asterias* ovum after fertilization seems to me unintelligible except as the result of an increase in permeability. Why it should be assumed that this increase is more than momentary, I fail to see.

IV. ANALYSIS BY MEANS OF THE RATE OF CLEAVAGE.

A. The Effect of Egg-Secretion in Normal and Alkaline Sea-Water.

A first step toward an answer however has been taken in the form of experiments in which known quantities of egg-secretion were added to normally fertilized eggs and the rate of cleavage compared with controls. The secretions used in these experiments were standard, prepared as before.

Many notes were taken in making these comparisons for it could not be foreseen which details might be utilized as indicators of the relative rates of development. Of all these however only certain data with respect to the 2, 4, and 8-cell stages are reported. This choice depends entirely on the advantage of reporting the more easily verifiable facts, and not because other details are in any way contradictory. Indeed the reverse is true and applies to such stages of the division spindles as can be clearly recognized in the living egg. Similar conclusions also can be drawn from the later stages, although it is much more difficult to tell at a glance whether a certain culture of blastulæ, gastrulæ or plutei, is more or less advanced than a given standard. I wish to emphasize the fact however that these later stages were obtained in large yields in the experimental cultures and were normal, though often somewhat slower of movement.

Several ways of comparing the rates of cleavage are open. Of these the following two were adopted: the time (minimum, in the table) that elapsed between insemination and the first 2, 4, or 8-cell stage seen in a particular culture is given, as well as the interval (maximum, in the table), between insemination and the time at which the cultures were at the height of these respective stages. The results of the second way of counting are in the same sense as the others, but are less accurate since there is greater opportunity for errors of judgment, whereas no error of judgment is likely with respect to the recognition of the first 2, 4, or 8-cell stage seen. The possibility that the particular cases observed were not the first to appear in the cultures of course remains, but a moment's examination of Table I will show that with very considerable errors of this sort consistent results could hardly have been obtained.

The observations were so made that intervals of one minute elapsed between the readings. Thus if *A*, *B*, *C*, *D* represent four culture dishes, *B* was read one minute after *A*, *C*, one minute after *B*, *D* one minute after *C*, and the second reading of *A* was taken four minutes after the first. Under such conditions one could introduce quite a large error from the mere arrangement of the dishes. This was guarded against both by reversing the order of reading from time to time, as well as by changing the order of the dishes in the several experiments. As a matter of fact the significant differences in my tables are always greater than the maximum error which might have resulted from the order of observation.

In the presentation of the results I have not compared the intervals for each of the three stages considered, separately (although the data for such comparison are given), but the average of the intervals that elapsed between insemination and the 2, 4, and 8-cell stages respectively. This procedure further eliminates errors attaching to any specific observation, besides reducing the number of comparisons necessary.

In the right hand division of the following table are contained comparable observations made in sea-water whose C_{OH} had been raised by the addition of 1.75 c.c. *N*/10 NaOH to 100 c.c. sea water. These experiments are included because they serve as checks on those in normal sea water. The theoretical considerations which prompted them were based on the fact brought forward by Loeb ('98) that the development of *Arbacia* is accelerated in alkaline sea-water, and depressed in acid. This is easily verified if normally fertilized eggs are allowed to develop to the blastula stage and are then divided into three lots, one for control, a second to which NaOH is added in the proportion of 1.75 c.c. *N*/10 per 100 c.c. of sea water, whereas the third is acidulated with HCl in the proportion of 1.75 c.c. *N*/10 per 100 c.c. of sea water. In such an experiment gastrulæ, with only here and there a short armed pluteus, predominate in the HCl culture at a given hour; the control at the same time contains a large number of plutei in various stages and some late gastrulæ, whereas the NaOH dish holds practically nothing but complete plutei. Loeb, in the paper referred to attributed this result to the probable acceleration of the oxidations due to the increase in OH ions. I used the NaOH in conjunction with the egg secretion on the theory that if the secretion affects the oxidations it should antagonize the effect of the NaOH, since the secretion alone, in the concentrations employed, retards the development. As measured by the first three cleavages however, the effects are not antagonistic, but additive, for the NaOH if anything had an effect exactly the reverse of the expected and that actually found for the later stages. This result is in harmony with Loeb's later work. In his recent book ('13^6), p. 35, he says: "The writer published years ago a paper in which he showed that the development of the eggs of *Arbacia* is retarded and finally inhibited if increasing quantities of acid are added to the sea water. He has since vainly attempted to show that the rate of development of the sea urchin egg can be increased with the increase of the concentration of hydroxylions in the sea water." If the reference here is to the paper cited above, evidence is there given that the increase in hydroyxlions does accelerate the development of *Arbacia*, but this acceleration was not noticed clearly until the day after fertilization. Both results seem really to be correct, only the rate of cleavage is either not accelerated or even depressed, whereas the rate of development from the blastula to the pluteus, is accelerated.

Table I.

Rates of Cleavage in Minutes After Insemination.

Cells.	Sea-Water 2 Vols.		Sea-Water 1 Vol. Secretion 1 Vol.		NaOH Sea-Water 2 Vols.		NaOH Sea-Water 1 Vol. Secretion, 1 Vol.		
	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	
2	44′	53′	47′	59′	45′	52′	54′	77′	Experiment I
4	64	74	73	92	52	74	72	93	
8	83	91	.....	.....	74	95	.....	.....	
2	48	52	47	70	53	80	51	70	Experiment II
4	48	71	55	83	80	.....	70	88	
8	71	84	63	88	85	.....	88	.....	
2	39	53	41	66	40	46	41	64	Experiment III
4	42	74	66	91	46	76	.....	77	
8	.....	92	95	.....	82	93	94	.....	
2	45	67	50	74	42	59	61	79	Experiment IV
4	48	72	50	88	44	86	73	109	
8	72	107	98	113	70	98	.....	.....	
Average rate....	55	74	62	82	59	76	76		for first three divisions
Average rate....	47	65	54	78	50	66	64	82	for first two divisions

It is apparent from these figures that whether we base our averages on the time that elapses between insemination and the minimum number of a given stage or the maximum, the results are always in the same sense. The special calculations based on the 2 and 4-cell stages have been made for all the columns in order to make fair a comparison of the first three with the last in which the records do not include the 8-cell stage. It seems reasonable to assert that the presence of egg-secretion in certain concentrations retards the early development of the *Arbacia* ovum, and that the presence of NaOH in definite concentrations increases the retardation.

B. The Effect of Egg-Extract in Sea-Water.

Experiments with egg-extract, corresponding to those with egg-secretion were made for the purpose of determining whether the egg contains, and is therefore possibly able to secrete during the brief period of increased permeability incident to fertilization, substances whose effect on the rate of development might be different from that of the secretions heretofore used. Extracts were made by the use of hypertonic sea-water ("double sea-water"), by grinding the eggs with pulverized glass as well as by laking them in distilled water. The quantities and concentrations used corresponded as nearly as possible to the amounts of secretion employed in the earlier experiments although other concentrations were tried. In all cases marked retardation of development was observed. Owing to the fact that the extracts prepared by the three methods are not identical and that their analysis has not been carried far enough as yet, accurate comparisons with the secretion are at this time impossible. However three characteristics of extract-cultures contrasted sharply with cultures developing in the presence of secretion. These were: numerous arrests of development in the early cleavages; much cytolysis; and a very general failure to get beyond the early non-motile blastula. These results indicate a qualitative difference between the secretion and the extract.

V. THEORETICAL.

The heightened rate of oxidation, the increased rate of secretion together with the decrease in volume on impregnation, all suggest as the essential point in the initiation of development, the relief of antagonistic conditions. When I found that the egg-secretion in certain concentrations actually retards development, I thought I had located the antagonists, and, without knowledge of the earlier and similar suggestion by R. S. Lillie ('09), postulated that initiatory agents are effective "because through increased permeability of the plasma film the egg is enabled to loose substances antagonistic to oxidation" ('13[3], p. 450).

It would of course have been more conservative to say "antagonistic to development," for the retardation brought about by the secretions and extracts may be the outcome of interferences with other conditions and processes, no less essential for normal development, than the oxidations. Indeed Loeb and Wasteneys ('11) have demonstrated the independence of the temperature coefficients of oxidation and cleavage, so that retardation of the latter is not synonymous with a depression of the former. Again the secretion, in the concentrations in which it was employed, brings about abnormal permeability relations which might account for the retarded development, and the possibility that the substances involved have one effect in the concentrations in which they occur in the unfertilized egg, but different effects in the higher concentrations of the experiments, must not be overlooked for good analogies are to be found in the effects of different concentrations of ether (R. S. Lillie, '12[2], p. 373). While it may yet be true that the delaying effect of the secretion is actually the outcome of a depression in the rate of oxidation, proof of this must be sought in further experiments.

However, even if further experimentation should succeed in tracing the retarded development to a decrease in the oxidation rate brought on by the presence of the egg-exudate, a difficulty would still remain, for the surface film of the unfertilized ovum is permeable for the secretion, and so by constant elimination an accumulation of the suspected antagonist to the inhibition point would be automatically prevented. This is the argument applied by Loeb in contraversion of R. S. Lillie's idea that CO_2 might be the antagonist, for inasmuch as CO_2 "is a good agency for calling forth membrane formation" and as only substances capable of diffusing into the egg can have this effect, the egg surface must be permeable to it before impregnation, and consequently there can be no accumulation that could be relieved as the result of the increase in permeability associated with fertilization.

However two possibilities remain; either the quantitative difference in the rate of secretion between unfertilized ova and ova undergoing fertilization is significant, or there is a qualitative difference as yet undetected between the secretions from the two kinds of eggs. Since "the velocity of segmentation in eggs fertilized by two spermatozoa is identical with that found in eggs fertilized by one. . . . ," we must believe, according to Loeb ('13[6], p. 13) "that the spermatozoan causes development . . . by removing an obstacle to development."

Certain facts and considerations to be dealt with in a later paper, seem to point to the possibility that in addition to the quantitative difference, the secretion at the moment of fertilization differs from the secretion of uninseminated eggs, qualitatively as well. Should further experimentation establish the correctness of this suspicion, then since the rate of oxidation in fertilized eggs is greater than in unfertilized, we could still say, "substances antagonistic to oxidation are eliminated at fertilization" although these substances may be neither CO_2 nor any of the constituents of the secretion from unfertilized eggs. Furthermore their action may be indirect through the inhibition of processes which when set going by their removal allow oxidation to proceed at the heightened rate normal for the fertilized egg. Whatever the final solution of these problems may be, it seems altogether likely that the initiation of development, and with it, the initiation of the processes leading to cell division, are in some way significantly related to the momentary increase in the permeability of the ovum, accompanying the process of fertilization.

VI. SUMMARY.

1. In corroboration of F. R. Lillie, it was found that the egg-secretions of *Arbacia punctulata* exert a chemotactic effect on sperm, and activate, agglutinate, and paralyze them.

2. The egg secretions of *Asterias forbesii* behave in a similar manner toward *Asterias* sperm.

3. *Arbacia* secretion activates, agglutinates, and paralyzes

Asterias sperm, and *Asterias* secretion has the same effects on *Arbacia* sperm.

4. Paralyzed sperm may be reactivated but not reagglutinated.

5. The egg secretions test negatively for reducing substances and do not give the usual protein tests although they were found to be faintly positive to the acid tests and the xanthoproteic. This may be due to traces of the egg jelly.

6. Agglutination may be gotten with dry egg powder.

7. The agglutination reaction very possibly depends on a surface effect.

8. More soluble substances escape from the egg of *Arbacia* in hypertonic sea-water and in sea-water infected with sperm, than from unfertilized eggs in normal sterile sea-water in the same length of time.

9. The egg-secretion in certain concentrations retards development measurably.

10. 1.75 c.c. $N/10$ NaOH added to 100 c.c. of sea water does not accelerate the early cleavages. The retardation noted may well be within the limits of error.

11. NaOH in the same concentration does markedly accelerate the development of blastulæ into plutei.

12. Egg secretion in the concentrations employed + NaOH in the concentration given above, results in a more marked retardation of cleavage than the egg-secretion without the NaOH.

13. Egg-extract, as contrasted with egg-secretion, in addition to retarding development, in a similar manner when employed in a similar manner, results in cytolysis, arrests of development in the early cleavages, and a general failure of the eggs to get beyond the early non-motile blastula.

14. The heightened rate of oxidation in the fertilized egg; the increased rate of secretion in eggs undergoing fertilization; the decreased volume after fertilization all point toward the possibility that initiation of development depends upon the removal of substances, directly or indirectly antagonistic to oxidation. Proof that the egg-secretion in certain concentrations measurably retards development is however insufficient evidence either for the conclusion that it itself is the antagonist, or contains it.

This would not follow even if it were shown that the retardation is the result of depressed oxidation. It is possible however that egg-secretion, at the moment of fertilization differs qualitatively from the earlier secretion, and contains the real antagonists whose inhibitory effect need not necessarily be thought of as having been direct.

15. These suggestions together with the facts upon which they are based, are not necessarily out of harmony with existing prominent theories of fertilization.

University of Michigan,
December 20, 1913.

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ON AUTO-PARTHENOGENESIS IN ARBACIA AND ASTERIAS.

OTTO GLASER.

(*From the Marine Biological Laboratory at Woods Hole and the Zoölogical Laboratory of the University of Michigan.*)

The unfertilized ova of *Arbacia* eliminate substances which discolor the sea-water, but when the same eggs are undergoing impregnation, the discoloration of an equal amount of sea-water in unit time, is roughly half again as much ('14[2]). Hand in hand with this fact goes the discovery that both *Arbacia* (*punctulata*) and *Asterias* (*forbesii*) ova, are measurably smaller in volume after fertilization than before ('13[1], '14[1]). These observations led me to begin an investigation which might throw some light, not only on the nature of the substances lost, but on the further question whether their elimination is significantly associated with the initiation of development. Not more than a beginning has been made, but some of the preliminary, chiefly qualitative, results are sufficiently interesting to be reported at the present time.

My earliest experiments consisted in a series of attempts to find what sort of substances could be gotten out of the eggs. I therefore prepared solutions in sea-water from ground, laked, or extracted ova. These preparations when tested with sperm exhibited the properties which had been described by F. R. Lillie for egg-secretion ('12, '13[2,3]). Emphasis must be laid on the power of these extracts to activate the sperm; on their chemotactic effect; and on their sperm-agglutinating as well as sperm-paralyzing capacities. Addition of the extracts in certain concentrations to normally fertilized eggs, resulted in a retardation of development; normal blastulæ instantly slowed their movements, and underwent a noticeable increase in volume when subjected to the extracts. Similar observations were made on the larvæ of *Arenicola* whose rate of movement was also slowed down, to be followed instantly by an outflow of their yellow pigment and a slight and reversible agglutination.

From these preliminary observations it appeared that the egg-extracts are capable of increasing the permeability of cells. On the view therefore that an increase in permeability is associated, significantly or otherwise, with fertilization, the initiation of development by means of these extracts should be possible. Against this idea stood the evidence of Loeb ('11) according to which "it was found that it was absolutely impossible to cause membrane formation of the sea-urchin egg by extracts from the sea-urchins of the same or even related species." From this Loeb concluded that the cortical layer of the egg exhibits against the lysins of the body producing it, the immunity characteristic for genetically related cells in general. It seemed little likely therefore that extracts prepared from the cells themselves should result in the initiation of development, and as a matter of fact, "membrane formation" in the sea-urchin egg did not occur. On the other hand, in corresponding experiments on the eggs of *Asterias*, a typical fertilization membrane did appear, and in both sea urchin and starfish, development was successfully initiated. For the type of initiation here dealt with, I have chosen the term auto-parthenogenesis.

Auto-Parthenogenesis in *Arbacia* by Means of Egg-Secretion.

The solutions employed in these early experiments, despite the use of weighed quantities of eggs, were not only very variable in concentration, but also differed in composition according to the method of preparation ('14^2). Since the secretion from unfertilized eggs, has the same effects on sperm as the extracts, it appeared possible that it might also have similar effects on blastulæ, *Arenicola* larvæ, and unfertilized ova. This indeed was soon found to be the case, although as pointed out elsewhere ('14^2) extract and secretion are probably not identical throughout.

The next step, the discovery of a more reliable mode of procedure, resulted in a definite method of securing results, but I have no reason for considering it final. Omitting the experiments of orientation and other unnecessary details, the following outline may be taken as a guide for further work: Standard secretion was prepared by adding to a certain number of "dry" ripe ovarian eggs, double their volume of sea-water. At the end of ten minutes, during which the eggs were slightly agitated at intervals, the suspension was centrifugated, and the eggs cast down. After 100 revolutions the supernatant fluid was carefully decanted and set aside for use.

Ripe eggs were then shaken, usually from the ovaries of a single individual, into a small quantity of fresh sea-water, and to 1 c.c. of a concentrated suspension of these, was added 1 c.c. of the secretion. In this mixture the eggs were allowed to stand 2 hours, when cleavages were usually found in all the dishes. That these were not the result of accidental infection with sperm was guaranteed by special precautions—all dishes, instruments and the hands as well as the animals used having been carefully sterilized. Furthermore, when the first urchin opened was a female, I usually sought no further, as the eggs from a single individual are sufficient in number. Indeed in all but two or three series only eggs from a single urchin were used. It might be urged that the secretion or the sea-water were infected. Sterilization of the latter however does not prevent auto-parthenogenesis, and against the idea that the sperm might have been present in the secretion there are three arguments, two of which are conclusive. In the first place, as has been shown by F. R. Lillie ('13^2) and myself ('14^2), the fertilizing power of sperm decreases markedly if they are allowed to remain in the secretion more than a few hours. In some of my experiments the secretion was prepared a day in advance, and used on the eggs of the first urchin opened on the following morning. In the second place fertilization membranes never appeared in any of the experiments with *Arbacia* eggs unless especially induced, and lastly, if the same eggs from which the secretion was prepared were afterwards subjected to its influence for the proper length of time and in the proper concentration, characteristic auto-parthenogenesis took place.

Many experiments were tried varying the concentration of the secretion as well as the time of exposure. My records indicate cleavages at higher concentrations as well as lower, and also in less than two hours, but the greatest number was always obtained when 1 volume of the concentrated egg suspension was exposed for 2 hours to 1 volume of the standard secretion. If at the end of this time the supernatant fluid is poured off and replaced by fresh sea-water, free-swimming blastulæ will be found within 24 hours. In one case only did development proceed to the pluteus stage.

Among the blastulæ that develop in these cultures are some characterized by an extremely slow rate of movement; others, though rare, may move at a practically normal rate, whereas the majority are considerably below normal in speed. This fact in earlier experiments misled me, as I caught sight of only the fastest swimmers, and mistook the others for arrested cleavages.

The size of the blastulæ is also a ready source of deception, since there are in general three kinds, micro-blastulæ, normal-sized blastulæ, and mega-blastulæ, of which the first are by far the most numerous. As these are usually very transparent and also slow of movement their presence in a culture may easily be overlooked.

The origin of the micro-blastulæ is clear. The first two cleavage cells, although normal in size, in most cases do not remain together, but separate and proceed to develop independently of one another. If the cells produced by these isolated blastomeres continue to separate very minute micro-blastulæ may result. Such falling apart of the cleavage products has been observed in the 16-cell stage and even later. The separation takes place, I believe, because the fertilization membrane has not appeared ('13[1]).

Normal-sized blastulæ need no explanation, since they must result from the eggs whose cleavage cells can be seen to hold together despite the absence of the normal fertilization membrane. As for the mega-blastulæ, it is possible that their origin is identical with that of mega-blastulæ in normal cultures. On the other hand, they were more noticeable in the auto-parthenogenetic series, and it is possible that the larger type of egg is better suited for this than the normal mode of development. Again the possibility is not excluded that the mega-blastulæ may come from eggs of normal size. This is not unlikely, since normal sized blastulæ instantly increase in volume when subjected to the same concentrations of the secretion.

Improved Method of Auto-Parthenogenesis.

Loeb's improved method of artificial parthenogenesis consists in following the treatment with parthenogenetic agents, by an after-treatment with hypertonic sea-water, 8 c.c. of 2.5 M NaCl + 50 c.c. sea-water. It seemed likely therefore that a better yield of larvæ could be secured if eggs, after having been subjected to the action of the secretion for two hours, were afterwards treated with the hypertonic solution for forty minutes. This surmise proved correct. The following is an outline of a typical experiment:

Time.	*A*	Time.	*B*
10.20 A.M.	1 c.c. eggs + 1 c.c. secretion.	10.12 A.M.	1 c.c. eggs + 1 c.c. secretion.
12.12 P.M.	Began hypertonic treatment.	1.12 P.M.	Began hypertonic treatment.
12.52	Ended hypertonic treatment.	1.52	Ended hypertonic treatment.
2.30	Considerable number of 2, 3, and 4-cell stages.	2.45	Many cleavages; 2–8 cells. Many normal.
5.45	Numerous 2, 3, and 4-cell stages.	6.00	Many 2, 4, 32, 64 and 128-cell stages.
	Very few 32–64-cell stages.	7.30	Cleavages in every field.
9.30 A.M.	1 active larva, many dead.		Some blastulæ but not free.
		9.45 A.M.	12 fine active larvæ.
			50 per cent. of cleavages arrested.
		4.00 P.M.	Hundreds of slowly moving blastulæ.

In experiment *B* the time of exposure to the secretion was three hours instead of two as in earlier experiments with the hypertonic after-treatment. From this one might infer that better results could have been obtained in the experiments in which the secretion alone was used, but the results do not warrant this assumption. Controls also show that with the hypertonic after-treatment, three hours of exposure to the secretion is better than two. We must believe therefore that the success of experiment *B* and all the others carried out in the same way, depended on the hypertonic after-treatment.

The differences in the length of exposure to the secretion are for the present purely empirical.

In all the experiments both with as well as without the hypertonic after-treatment, development was much delayed in many of the eggs, and rarely went beyond the blastula stage. As stated before, I observed plutei only once, and not over ten or twelve at that. Gastrulæ also were rare. For this failure to pass beyond the blastula stage there are several reasons; in the first place one must attribute a large share to the fragmentation of the early cleavage stages, due as I believe to the absence of a normal fertilization membrane, and secondly the abnormal permeability relations resulting from the treatment with the secretion probably also play a rôle ('14[2]) by placing mechanical difficulties in the way of gastrulation even though the necessary materials are present in the embryo. The evidence for this conclusion cannot be presented now. Although these obstacles stand in the way of perfecting this method of rearing sea-urchins, the fact that it is impractical, and inferior to other methods does not in the least detract from the theoretical interest of the results. As McClendon ('09) puts it: "In natural parthenogenetic development the end result may be a maturation or segmentation stage or a larva or adult. Though only the reproductive adults are of significance to the species, all are of significance to science."

Auto-Parthenogenesis in *Asterias*.

In earlier papers ('13[1], '14[1] and '14[2]) I have emphasized certain differences as well as certain points of similarity between the eggs of *Arbacia* and *Asterias*. Chief among the differences, are the characteristics of the fertilization membranes; most important among the points of likeness are the decrease in volume to be observed in both kinds of eggs on fertilization, and the fact that the *Asterias* ovum produces a secretion in which the most striking characteristics of the corresponding *Arbacia* exudate can be verified point for point. For these reasons one might anticipate the initiation of development in the *Asterias* egg by the proper employment of its own secretion.

In the following experiments the secretion was prepared exactly as in the case of *Arbacia*, but the eggs before treatment were given time to mature, *i. e.*, they were allowed to stand for about 1 hour in sea-water after removal from the ovaries.

10.27 A.M.	1 volume *Asterias* eggs. 1 volume *Asterias* secretion.	10.27 A.M.	2 volumes *Asterias* eggs. 1 volume *Asterias* secretion.

10.50	Fertilization membranes indicated.	10.53	Fertilization membranes indicated.
12.15 P.M.	Many eggs elongated.		
2.00	Fertilization membranes. Frequent cleavages.	2.00 P.M.	Frequent cleavages.
9.00 A.M.	Cytolysis. One free swimming blastula.	9.00 A.M.	Numerous dead blastulæ. Much gastrulation.

In other tests similar results were obtained. Unfortunately this work came at the end of the *Asterias* breeding season, and further experiments were impossible. Those performed however are sufficient to show that parthenogenesis can be induced in *Asterias* as in *Arbacia* by the use of its own egg-secretion. Owing to the existence of a perivitelline space within which the cleavage products of the *Asterias* egg are held, it appears likely that better results will ultimately be achieved than in the auto-parthenogenesis of *Arbacia*. In fact as indicated above, even without hypertonic after-treatment, which in first experiments was always omitted for the sake of control, one of my auto-parthenogenetic *Asterias* cultures proceeded to the gastrula stage.

"HETERO-PARTHENOGENESIS."

"Hetero-parthenogenesis" as the sequel will show, is probably not the correct word, but I use it here as a convenient heading for experiments in which development was initiated in *Asterias* eggs by the use of *Arbacia* secretion. Such experiments suggested themselves, as the result of the earlier observations on the increase in the permeability of various types of cells. From these one might anticipate "hetero-parthenogenesis," a process which was indeed successfully induced as the following experiment indicates:

10.35 A.M.	1 volume mature *Asterias* eggs. 1 volume *Arbacia* secretion. No immediately visible effect.
10.50	Fertilization membranes indicated.
2.00 P.M.	Numerous cleavages.

THE FERTILIZIN THEORY.

After I had discovered auto-parthenogenesis through the observation that the egg-extracts are permeability increasing agents, I consulted with Professor F. R. Lillie who had been performing those experiments on normal fertilization in *Arbacia* which have led him to propose the fertilizin theory, briefly outlined in his paper "The Mechanism of Fertilization" ('13^3).

The facts on which this theory is based are dealt with in F. R. Lillie's earlier papers, as well as in the one specifically referred to. In my paper on "A Qualitative Analysis of the Egg-Secretions and Extracts of *Arbacia* and *Asterias*" ('14^2) will be found certain verifications of Lillie's observations. It would take us too far to go into the factual basis of the theory here, but briefly it postulates as the essential point in fertilization a chain of chemical reactions in which an amboceptor-like substance—the sperm-agglutinating agent—present in the secretions from the egg, unites by means of a spermophile side-chain with receptors in the sperm, and by an ovophile side-chain with receptors in the egg.

If this chain of reactions indeed occurs and is related to fertilization and the initiation of development in the significant manner put forward by Lillie, it follows that there are theoretically at least five possibilities of blocking fertilization. Lillie has listed these as follows:

1. Through loss of fertilizin by the egg.
2. Through occupancy of the sperm receptors.
3. Through occupancy of the egg receptors.
4. Through occupancy of the ovophile side chain of the amboceptor (fertilizin).
5. Through occupancy of the spermophile side-chain of the amboceptor (fertilizin).

Of these Lillie has proved possibility 1, by washing the eggs; 4, by means of an inhibitor in the blood which prevented fertilization, but not the sperm-agglutinin reaction; and 5, by setting free from the egg itself an inhibitor, the anti-fertilizin which obstructs the union of the sperm with the amboceptor by occupying the spermophile side-chain. Since therefore the presence of the agglutinating agent, and moreover its presence in a state in which both spermophile and ovophile side-chains are free to combine with their respective normal receptors in the sperm and the egg, seems to be necessary for normal fertilization, Lillie has called the substance in question, fertilizin.

F. R. Lillie has also offered an interpretation of my results on auto-parthenogenesis. He suggests that in the initiation of development by employment of the egg-secretion, essentially the same chemical chain is involved as in normal fertilization except that the sperm, and naturally the sperm-fertilizin reaction also, is dropped. In other words, in auto-parthenogenesis, the egg-receptors are thought of as combining with the ovophile side-chains of fertilizin which has not been bound to sperm-receptors through its spermophile groups.

Off-hand this supposition does not appear unreasonable. The fertilizin is certainly present in abnormally high concentration in the secretion as used for auto-parthenogenetic initiation, and the mere fact that the secretion is a permeability-increasing agent does not preclude the possibility that one of its constituents may play an additional rôle in the initiation of development. If this is true, it should be possible to block auto-parthenogenesis in accordance with the scheme outlined above, although the absence of the sperm and the sperm-fertilizin reaction restricts the opportunities to 1, the loss of fertilizin; 3, the occupancy of the egg-receptors; and 4, the occupancy of the ovophile side-chain of the fertilizin.

Methods of Preventing Auto-Parthenogenesis.

1. *The Prevention of Auto-parthenogenesis by Washing the Eggs.*—Several preliminary experiments in which the eggs before being exposed to the action of the secretion were washed from 20 minutes to half an hour in great excesses of sea-water, proved conclusively that this procedure very materially reduces the number of cleavages, and the number of larvæ, afterwards found in the cultures. These results led to a more careful test carried out on eggs which had been washed in excesses of sea-water for 24 hours. The sea-water was changed frequently but at irregular intervals. These eggs were then treated in the usual way with the secretion, but not a single cleavage was found. Thus the effect of even slight washing is noticeable whereas prolonged washing effectually prevents the auto-parthenogenetic cleavage. From this we might conclude that something essential for the initiation of development had been removed from the eggs.

However the failure of the fertilizin with which the eggs were treated and for which they are permeable, to offset the effects of washing, remains to be accounted for.

2. *The Prevention of Auto-parthenogenesis by Means of a Group Split from the Sperm.*—Lillie has made the apparently justifiable assumption that fertilizin bound by sperm is activated, *i. e.*, has a greater chemical affinity for the substances with which it unites in the egg than the unbound fertilizin. If this is true, it occurred to me that the use of sperm, in conjunction with the secretion might give better auto-parthenogenetic results than had been obtained by the use of the secretion alone, either with or without the hypertonic after-treatment. Naturally only dead or seriously injured sperm are available for this experiment, and with the latter one could not be sure to exclude a certain and misleading number of real fertilizations which would then be mistaken for very fine cases of parthenogenesis. Since the power of the fertilizin to produce the agglutination reaction and to play a part in normal fertilization is not destroyed by brief boiling, it seemed possible that the complementary chemical groups in the sperm might also survive boiling without being destroyed or so altered as to be incapable of taking some part at least in the reactions between egg and fertilizin.

However, instead of furthering auto-parthenogenesis, exactly the opposite effect was found, for the boiled sperm absolutely prevented the process, as shown by the following experiment:

1 volume of eggs + 1 volume of secretion[1] + 2 volumes boiled sperm infusion 10.40 A.M. to 2.50 P.M. Four hours later little if any effect visible; a few cell-masses were noted, but nothing that in any way suggested auto-parthenogenesis. At 2.58 P.M. the mixture was diluted with an excess of sea-water. At 6 P.M. no effect was noticeable; by 10 A.M. the next day, the eggs had undergone a granular decomposition.

Experiment repeated carefully controlled. Result identical. In the control however auto-parthenogenesis occurred and on the following morning 12 very fine larvæ were seen in addition to the usual number of delayed cleavages and microblastulæ.

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[1] Unfortunately the egg-secretion in these tests was more dilute than usual, but the effect noted is not interpretable on this basis since parthenogenesis occurs at greater dilutions. The sequel to these experiments further emphasizes the interpretation given here.

Since the fertilizin theory postulates essentially the same machinery for auto-parthenogenesis and normal fertilization it follows that initiation by living sperm should also be blocked by treating the eggs with the boiled sperm-infusion. This is actually the case:

> Eggs treated for two hours with strong boiled sperm-infusion. At the end of this period they were heavily inseminated with living sperm, but remained unfertilized. Only a few cleavages were observed later, but these were arrested at the latest in the four-cell stage. No further development occurred, but on the following day practically all the eggs exhibited fertilization membranes. Controls normal.

3. *The Prevention of Auto-parthenogenesis by Means of a Group Split from the Secretion.*—F. R. Lillie ('13²) finds the agglutinative power of egg-secretion unimpaired, or perhaps better, not measurably impaired, by boiling for 5 minutes, although the solution undergoes a change of color due to the formation of small quantities of a purple compound ('14²). As the secretion is thus visibly different in chemical composition it becomes important to ascertain whether the formation or presence of the purple compound makes any recognizable difference in the initiation of development. The following experiments indicate that the efficacy of the secretion as a parthenogenetic agent is blocked.

Standard secretion boiled. Purple compound formed. Sperm-agglutination positive.

3.20 P.M. 1 volume of boiled secretion added to 1 volume of fresh egg-suspension.

8.00 P.M. No cleavages.

8.00 A.M. Few delayed cleavages, not at all in normal frequency.

11.30 A.M. 1 volume of boiled secretion added to 1 volume of fresh egg-suspension.

2.00 P. M. No cleavages.

7.00 P.M. No cleavages.

8.30 A.M. No cleavages.

Control in unboiled secretion exhibited auto-parthenogenesis in usual quantity.

INTERPRETATION OF EXPERIMENTS ON PREVENTION OF AUTO-PARTHENOGENESIS.

Analysis of the means by which a process can be prevented may be even more suggestive than a similar analysis of the means by which it can be induced, and this proves to be true in the present case. How then are these experiments to be understood?

1. *The Block in Washed Eggs.*—The prevention of fertilization by washing (F. R. Lillie, '13[3]), according to the fertilizin theory, is due to the absence of the intermediary body with which both sperm and egg receptors normally unite. We cannot, however, despite the superficial similarity of the two cases, attribute the auto-parthenogenetic block to the absence of suitable bonds for the egg receptors since the surface film of the ovum is perfectly permeable for fertilizin. This must diffuse into the eggs from which it has been removed the moment they are exposed to the concentrations employed for initiation. If this is correct, then the prevention of auto-parthenogenesis in washed eggs must be explained in some other way. This however is not necessarily fatal to the fertilizin theory, for these same experiments strongly suggest that a mere increase in permeability is insufficient to initiate development, a suggestion still further reinforced by the second type of block to be considered presently.

The experiments with washed eggs are also suggestive from another standpoint. Lillie (*loc. cit.*) tells us: "If it be true that the egg contains its own fertilizing substance it might also be possible to induce parthenogenesis by increasing the concentration of this substance to a certain point. . . ." The washed eggs however demonstrate that the fertilizin experimentally added to the solution about the eggs has no fertilizing effect, and if it has none when the eggs have been washed, there is no good ground for considering its effect different when the eggs have not been washed. "Hetero-parthenogenesis" strongly backs up this position. It appears most likely therefore that in auto-parthenogenesis the fertilizin experimentally added, plays the same rôle as any other permeability increasing agent effective in the artificial initiation of development. If this is correct, and furthermore, if the parthenogenetic block in washed eggs is not identical with the fertilization block brought about by the same means, it follows, not that the fertilizin theory is inapplicable to these cases, but only that it does not apply exactly as F. R. Lillie has supposed.

2. *The Block by Means of Groups Split from the Sperm and the Egg-Secretion.*—The prevention of both fertilization and auto-parthenogenesis in eggs previously treated with boiled sperm infusion, as well as the parthenogenetic block in eggs subjected to the action of boiled secretion, seem at first sight to have little in common. In reality however there is an element of identity in all three cases, for suspensions of sperm as well as the egg-secretion give on boiling a characteristic color reaction due to the formation of a purple substance which may be looked upon as a chemical cleavage product split off from a mother substance. In an earlier paper ('14[2]) I suggested on other grounds, and in complete ignorance of its rôle in parthenogenesis, that this purple material might prove to be significant, as it seems to be specific for *Arbacia.* This suggestion now appears almost certainly correct, and I therefore propose for this substance, provisionally at least, the name "Purple X."

An attempt to understand the part played by Purple X in these reactions must begin with the proof that this substance and not some other cleavage product is really the effective one. Such proof would be found if the block to both parthenogenesis and fertilization could be removed with the removal of the Purple X. To accomplish this and at the same time not render the solutions toxic by the addition of precipitating agents, called for methods which had first to be discovered.

The Purple X is either in a state of very fine suspension, bordering on solution, or is actually in solution. Since now a precipitable colloid frequently carries other bodies with it when thrown down, I added the albumen of hen's egg to the *Arbacia* egg-secretion, and boiled the mixture. The expectation that the purple compound might be removed at least to a considerable extent, proved correct, as the white coagulum of hen's egg albumen was quite markedly discolored by the purple substance. After filtration the secretion was tested for its parthenogenetic effect. The method of separation however is not quantitative and so the results were not perfectly clear cut. Nevertheless it remains true that the only boiled secretion that ever induced parthenogenesis was one from which the Purple X had been removed by coagulating white of hen's egg. This result, I may add parenthetically was obtained only after a very remarkable effect of the egg-white itself had been investigated. Even in the dilutions in which it is present in the sea-water after boiling, the white of a hen's egg produces very regular and curious distortions of the eggs which may even be divided into pseudo-cleavage products. However the effect does not seem to be analogous to parthenogenesis, and I shall therefore deal with it separately at another time.

In the course of these experiments a very much better and simpler method was found, for the Purple X is a relatively unstable compound and readily disappears. Thus a concentrated sperm-suspension boiled on one day and giving a color as deep as port wine, may on the next be golden yellow. With such a solution eggs were treated for one hour and five minutes. At the end of that time the addition of living sperm resulted in the fertilization of practically every egg. The experiment was repeated, the eggs being exposed for two hours and forty minutes, and again fertilization occurred.

As a counterpart to these experiments with sperm, the parthenogenetic effect of boiled secretion which had lost its purple color was tried. Such solutions contrast sharply with the newly boiled secretion, for whereas the latter very effectually blocks auto-parthenogenesis, development can be induced without difficulty after the spontaneous disappearance of the Purple X.

Since the Purple X-block occurs in the presence as well as in the absence of sperm, we can be certain that it is not the result of an effect upon the male sex cells or, in the language of the fertilizin theory, of occupancy of the sperm receptors. Which particular one of the remaining three blocks theoretically possible we are dealing with however must for the present remain an open question, although if the immediate effect of the egg-secretion is really identical with the immediate effect of any other parthenogenetic agent, occupancy of the egg-receptors is indicated. If this is true, then Purple X should block parthenogenesis no matter how induced. That this process should be blocked by less than is required to prevent fertilization is perfectly intelligible on Lillie's assumption that sperm-bound fertilizin is activated and has a greater affinity for the egg-receptors than free fertilizin.[1]

An Attempt to Construct a Provisional Working Hypothesis.

Although no final interpretation of the phenomena of fertilization is possible at the present time, an attempt to formulate some viewpoint which does no violence to our knowledge, and which at the same time may serve as a working hypothesis, must be made for further guidance.

If we accept the evidence of F. R. Lillie, together with my verifications and additions to it, and remember that many of the facts discovered by Lillie, as well as some found by myself, are the direct products of the fertilizin theory considered as a tool, I think we may even now admit that it is a useful working hypothesis. Lillie ('13) himself has emphasized this point, and moreover has listed among the other advantages of his theory, that it gives us an explanation of the specificities of fertilization; may furnish the foundations for the chemical conceptions needed by any theory of fertilization, and above all, that it offers one explanation for the initiation of development, whether by fertilization or parthenogenesis.

To what extent these claims are justified, and to what extent the theory will undergo modification, it would be unprofitable to discuss at present. One claim and its consequences however does invite discussion at the present time, and in this connection, for if the fertilizin theory is really capable of unifying the various methods of inducing parthenogenesis, its relations to another view, which apparently does the same thing must be analyzed, and furthermore we must answer the question whether all cases of parthenogenesis are really instances of auto-parthenogenesis.

[1] Since Purple X also appears when spermatozoa come into contact with the secretion, it is possible that its appearance plays a rôle in the prevention of polyspermy. This would not prevent a similar effect on the part of the anti-fertilizin, as suggested by F. R. Lillie. Indeed the chances that any particular sperm will complete the reactions postulated by the theory, must be exceedingly small when we reflect on the immense numbers which collect about each egg. Such collections become more intelligible when we recall that whereas the biparental effect can be carried out by a single sperm, the initiatory effect, at least in *Arbacia* ('13[1]) requires more than five.

1. *The Surface-Alteration Theory.*—The view which we may call the surface-alteration theory is a product of the work of Loeb, R. S. Lillie, and many others. According to Loeb the initiation of development depends in the first place upon a superficial cytolysis, or destruction of the cortical layer of the egg, brought about either by a lysin in the sperm, or by the so-called parthenogenetic methods and agents. After this alteration of the surface the rate of oxidation in the egg is raised from four to six times. Loeb says:

"There are two possibilities by which this result can be produced: either a catalyzer (an oxidase) is carried into the egg by the spermatozoon; or the change in the surface layer itself causes the increased rate of oxidation. Everything speaks in favor of the second assumption."[1] Further on Loeb suggests "that the spermatozoon causes development . . . by removing an obstacle to development."[2]

R. S. Lillie ('11 and earlier papers) has suggested that the superficial cytolysis increases the permeability of the egg, and further that through this increase in permeability a substance antagonistic to oxidation is eliminated from the egg. This substance he imagined might be CO_2 ('09).

Against this idea Loeb has raised objections. Inasmuch as CO_2 "is a good agency for calling forth membrane formation" and as only substances capable of diffusing into the egg can have this effect, the egg surface must be permeable to CO_2 before fertilization, and consequently there can be no accumulation that could be relieved as the result of an increase in permeability.

This criticism seems valid, and while it constitutes a reason for giving up the idea that the antagonist lost is CO_2, it in no way bears on the other idea, namely that there is an increase in the permeability of the ovum as the result of fertilization. However this idea also has failed of acceptance by Loeb, but this is due chiefly I believe because the evidence in support of it has been unfortunate not only in a purely technical sense which has been justly criticized, but also because it was gathered under a misapprehension apparently as to exactly what R. S. Lillie had suggested. So far as I can see, experiments in which the permeability of fertilized eggs is compared with that of unfertilized have no bearing on the question, for no such difference was postulated, but only that the "increased permeability is . . . temporary in normal or in favorable parthenogenetic fertilization" ('11, p. 307).

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[1] "Artificial Parthenogenesis and Fertilization," 1913, p. 13.

[2] The obstacle "removed" is a cortical layer unsuited for development, and the "removal" is in reality an alteration of this cortex: "Through cytolysis of the cortical layer of the egg the oxidations in the unfertilized egg are accelerated from four to six times their normal rate" (loc. cit., p. 14). Since this heightened oxidation persists after fertilization, and since the permeability of fertilized eggs may not be different from that of unfertilized, it is possible that the cytolysis of the cortical layer alone is insufficient to account for the increase in the rate of oxidation.

If this idea is correct we might expect two things to happen at fertilization in the egg of *Arbacia.* Since this egg before impregnation actively secretes materials that discolor the sea-water, the discoloration produced by eggs undergoing fertilization might be greater. I have found ('14[2]) that it is one and a half times that of eggs not undergoing impregnation. Further, fertilized eggs, after the process is complete, do not discolor the sea-water. In the second place even a temporary increase in permeability should result in a change in volume if the eggs remain in a medium that remains constant during the period under discussion. Measurements show ('14[1]) that the eggs of both *Arbacia* and *Asterias* are smaller in volume after fertilization than before.

I therefore consider the idea of increased permeability *during* fertilization correct, and that we may suppose all cases of fertilization, whether with sperm or without to have this one point in common. Since many of the results now published had not been found at the time I wrote my preliminary paper ('13[1]) I threw emphasis on this common attribute in section VIII. This emphasis I do not wish to withdraw as the result of later work, for this has only strengthened my conviction that in fertilization an increase in the permeability of the ovum actually occurs. The only question at issue seems to me to be the significance of this change for the initiation of development.

On this particular point I also postulated the loss of substances antagonistic to oxidation. This suggestion was based on the fact that substances are lost during the act of fertilization at a higher rate than before, and finally that the presence of egg-secretion in certain concentrations retards development. The retardation I supposed to be due to the depression in the rate of oxidation. This assumption, as I pointed out, does not seem unreasonable; on the other hand as I pointed out more recently, retardation of development may in this case also be due to interference with, or depression of, other processes no less essential. Only further work can decide these questions.

2. *The Fertilizin Theory.*—In the fertilizin theory, no particular rôle is assigned to the increased permeability. "The nature of the effect of the activated fertilizin on the egg is analogous in some respects to a superficial cytolysis, in this respect agreeing with Loeb's theory. But the 'lysin'[1] is contained in the egg, not in the sperm as Loeb thought; if cytolysis is involved, it is a case of auto-cytolysis. This may involve increase of permeability, the effects of which R. S. Lillie especially studied" ('13^3). These possibilities were mentioned "in order to point out that the conception" of the fertilizin theory "is not in conflict with the well-established work of others." This is very important for the fertilizin hypothesis is based on certain facts which seem to permit the interpretation given them, and the surface-alteration theory also has a verifiable factual basis. These facts, occurrences in and about fertilization, cannot be in conflict with one another. The problem is to understand their interrelations. The experiments on auto-parthenogenesis carry us a step forward in this direction.

At first sight success in initiating the development of *Asterias* eggs by means of *Arbacia* egg-secretion would appear to seal the death-warrant of Lillie's fertilizin theory. On the other hand a little reflection will show that no radical conclusions need be drawn. Parthenogenesis, as is well known can be induced in different eggs by the most diverse means—electricity, heat, cold, mechanical shock, osmotic pressure,[2] specific chemical alteration of the surface film or cortical layer, lipoid solvents, and even the prick of a needle having been found effective. These methods, each of which is capable of variation and modification, have nothing in common except their result, and it is idle to try to imagine any direct causal connection between them and the result. However one effect, common to all, seems to stand intermediate between the parthenogenetic result, and the experimental means used to induce it. This common intermediary is a change in the permeability of the surface of the eggs, and in the experiment in which parthenogenesis is induced in one ovum by the secretion of another it is not necessary to assume that anything occurs that does not also take place in all the other cases. The same reasoning applies also to auto-parthenogenesis, a process which was found by application of the surface-alteration theory, and in total ignorance of the fertilizin hypothesis.

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[1] It would have been more correct to say: it *was* contained in the egg.

[2] In the case of the purely osmotic methods, Loeb supposes that "the hypertonic solution acts simultaneously in two capacities: first as a cytolytic agent causing a change in the cortical layer (the formation of a gelatinous film) and second, as a corrective agency" ('13^4, p. 159).

The cytolytic action of the hypertonic solution is not without evidence, and so we may consider this method as having the effect common to all the others. If however the increase in permeability is effective also on account of the destruction of the normal fertilization block, in the sense outlined in the text, loss of water by the egg would directly or indirectly accelerate the loss of the antagonists (cp. Glaser, '13, p. 450).

Now the picture of fertilization with sperm given by the fertilizin theory is briefly the sperm-receptor-amboceptor-egg-receptor reaction. In the picture of auto-parthenogenesis, the sperm is dropped, leaving the amboceptor-egg-receptor to react, the former with an unsatisfied bond. Since however parthenogenesis can be induced without the addition of fertilizin to the eggs there is no reason for supposing that the egg-receptors united with the fertilizin which was added in my experiments for the purposes of initiation. This however does not seem to me to be at all necessary, for both substances are present in the egg from the beginning and we may postulate a union between the receptors and the fertilizin within the egg.

If this is indeed the mechanism of both parthenogenesis and auto-parthenogenesis, we must answer the question why the egg was not fertilized before, or what amounts to the same thing, why fertilization occurs at all.

In speaking of parthenogenetic agents, Lillie (*loc. cit.*) tells us, they "need only remove obstacles to the union of the amboceptor and the egg-receptor" (p. 527); and in connection with auto-parthenogenesis, that this might result "if the concentration of fertilizin were raised to a certain point, though it is conceivable that no increase in concentration would break down the resistance that normally exists to the union of the amboceptors and egg-receptors" (527). In other words, the egg is not always fertilized, because there are obstacles or resistances. When these are removed, fertilization, parthenogenesis, or auto-parthenogenesis may occur.

No circumstance connected with the initiation of development that is not a common occurrence in all cases is capable of explaining how these obstacles or resistances can be removed. It is not necessary for clear thinking on this subject to know what they are at present, but the only common fact which seems capable of explaining how these natural blocks to fertilization, and the initiation of development could be removed, is at present the increase in the permeability of the ovum. For this reason, without in any way trying to minimize the significance of the facts emphasized by the fertilizin theory, I believe that the permeability change plays a very real part in the process of fertilization, for through it is possible a disturbance of the equilibrium in which we know the egg finds itself prior to impregnation or parthenogenetic initiation.

If this viewpoint is correct, the following picture of both fertilization and parthenogenesis is possible. Fertilizin alone in sufficient concentrations is capable of increasing the permeability of the ovum; smaller quantities when activated by union with sperm are able to exert the same effect. This alteration of the surface may also be brought about by electricity, heat, cold, mechanical shock, specific chemical alteration of the membrane, lipoid solvents, pricking, or any other way of rendering the egg surface more permeable or of accomplishing the same result as an increase in permeability. The immediate effect of this permeability increase is a removal of those obstacles or resistances which constitute the normal fertilization block. These removed, the fertilizin within the egg combines with the egg-receptors, but if fertilizin activated by sperm is present, the egg receptors would combine with the elements for which ex hypothese they have the greater affinity.

So far the facts seem to group themselves harmoniously, but how are we to conceive of the "obstacles" or "resistances"? We might imagine the existence within the unfertilized egg of a body capable of playing the required rôle, but for this assumption there is no evidence. Moreover the substances demonstrated by F. R. Lillie, seem to me sufficient to account for the normal fertilization block if we make a much simpler assumption—one indeed which appears necessary on chemical grounds.

The ovum, according to Lillie, contains egg-receptors, fertilizin, and antifertilizin. Chemical affinities exist between the anti-fertilizin and the spermophile groups as well as between the receptors and the ovophile groups. Although Lillie does not postulate a reaction involving these elements in the unfertilized egg, it is difficult to believe that three substances, demonstrably capable of uniting in the manner indicated, would lie side by side without entering into combination, however loose. If this assumption prove valid, we can consider this union as constituting the normal fertilization block ("resistance," "obstacle") of the unfertilized egg.

From this standpoint it appears possible to explain the block to parthenogenesis in washed eggs. The egg surface prior to fertilization or treatment with parthenogenetic agents, is perfectly permeable to fertilizin. Lillie has shown, however, that the anti-fertilizin can only be removed by destruction of the ova, whose surfaces, with respect to these two substances, appear to act like dialysers. It is quite likely therefore that washed eggs differ from unwashed by a relatively higher concentration of anti-fertilizin. If now fertilizin is permitted to diffuse into a washed egg, it would be bound by the anti-fertilizin and so an initiatory effect, due to its further union (if indeed this occurs[1]) with the egg-receptors would be prevented. According to Lillie's view and the law of mass action, auto-parthenogenesis might occur in washed eggs when the concentration of fertilizin is more than enough to bind the anti-fertilizin. That it takes about two hours to initiate auto-parthenogenesis, and that I failed to initiate it in that length of time in washed ova, are both in harmony with the above suggestion.

So far as I can see, this is the only way at present, in which it seems possible to draw a picture in which all the facts and all the cases may find a place. It follows from the preceding reasoning, as well as more directly from the experiments themselves, that the rôle of the egg secretion (fertilizin) experimentally added to induce development was exactly the same as the rôle of any other parthenogenetic agent, and that it united with the egg receptors, no more than do the electricity, heat, cold, mechanical shock, osmotic pressure, or what-not. On the other hand, like these, the egg-secretion by affecting permeability, brings about conditions under which the ovum is freed from its inhibitors and, in a sense, enabled to fertilize itself. From this standpoint all parthenogenetic methods are methods for inducing auto-parthenogenesis, and fertilization with sperm differs from auto-initiation simply in the fact that the fertilizin which unites with the egg receptors has itself previously united with the sperm in the jelly, or the water about the egg.[1]

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[1] The avidity of fertilizin bound by sperm for the egg-receptors is assumed by Lillie to be greater than in unbound fertilizin. The opposite effect, resulting from a union of the spermophile groups with the anti-fertilizin is not excluded.

Summary.

1. The egg-extracts as well as the egg-secretions of *Arbacia* and *Asterias*, in addition to characteristic effects on spermatozoa, are permeability increasing agents, and initiate development. For the type of initiation here dealt with I have chosen the term *auto-parthenogenesis*.

2. Auto-parthenogenesis is blocked in *Arbacia* by washing the eggs in sea-water.

3. A group "Purple X" which may be split off from the sperm of *Arbacia*, and which also appears in boiled egg-secretion, blocks parthenogenesis in low concentrations and fertilization in high concentrations.

4. Prevention of parthenogenesis by washing can hardly be the result of an absence of fertilizin; prevention by Purple X may be the result of occupancy of the egg-receptors.

5. It is not unlikely that all cases of parthenogenesis are in reality instances of auto-parthenogenesis.

6. With certain minor modifications it appears possible to

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[1] According to F. R. Lillie's account, the sperm unites with the fertilizin in the egg. This however seems incredible, for the affinity between sperm and fertilizin is very great, and since the egg jelly, through which the sperm must pass on their way to the ovum is heavily charged with the fertilizin, it is difficult to see how any unbound sperm could reach the egg. This change in the geographical location of the sperm-fertilizin reaction however makes absolutely no difference in essentials.

construct an hypothesis of fertilization in which the basal facts of the surface-alteration theory, as well as the facts emphasized by the fertilizin theory may, without violence to our knowledge, find a place. From the standpoint here adopted, there is no antagonism between the two theories which emphasize different groups of facts associated with the process of fertilization. These facts cannot be in conflict with one another.

University of Michigan,
January 10, 1914.

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